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(71) Applicant (for all designated States except US): GENENTECH, INC. [US/US]; One DNA Way, South San Francisco, CA 94080 (US).

## (72) Inventors; and

(75) Inventors/Applicants (for US only): BACA, Manuel [AU/US]; Apartment #H3, 888 Foster City Boulevard, Foster City, CA 94404 (US). WELLS, James, A. [US/US]; 1341 Columbus Avenue, Burlingame, CA 94010 (US). PRESTA, Leonard, G. [US/US]; Apartment 206, 1900 Gough Street, San Francisco, CA 94109 (US). LOWMAN, Henry, B. [US/US]; 400 San Juan Avenue, El Granada, CA 94018 (US). CHEN, Yvonne, Man-Yee [CA/US]; 1951 O'Farrell Street #321, San Mateo, CA 94403 (US).

(74) Agents: DREGER, Walter, H. et al.; Flehr, Hohbach, Test, Albritton &amp; Herbert LLP, Suite 400, 4 Embarcadero Center, San Francisco, CA 94111-4187 (US).

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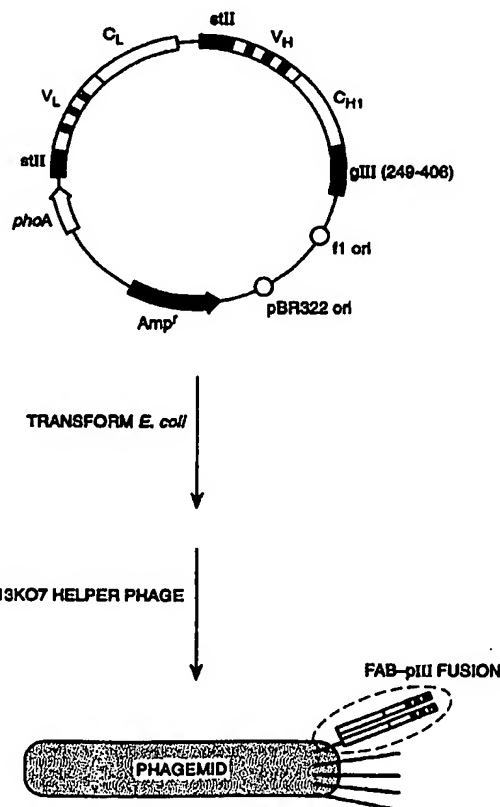
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## (57) Abstract

Humanized and variant anti-VEGF antibodies and various uses therefor are disclosed. The anti-VEGF antibodies have strong binding affinities for VEGF; inhibit VEGF-induced proliferation of endothelial cells *in vitro*, and inhibit tumor growth *in vivo*.



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## ANTI-VEGF ANTIBODIES

### Cross References

This application is a continuation-in-part of co-pending U.S. Application No. 08/833,504, filed April 7, 1997, which application is incorporated herein by reference and to which application priority is claimed under 35 U.S.C. §120.

## BACKGROUND OF THE INVENTION

### Field of the Invention

This invention relates generally to anti-VEGF antibodies and, in particular, to humanized anti-VEGF antibodies and variant anti-VEGF antibodies.

### Description of Related Art

5 It is now well established that angiogenesis is implicated in the pathogenesis of a variety of disorders. These include solid tumors, intraocular neovascular syndromes such as proliferative retinopathies or age-related macular degeneration (AMD), rheumatoid arthritis, and psoriasis (Folkman *et al. J. Biol. Chem.* 267:10931-10934 (1992); Klagsbrun *et al. Annu. Rev. Physiol.* 53:217-239 (1991); and Garner A, *Vascular diseases. In: Pathobiology*  
10 *of ocular disease. A dynamic approach.* Garner A, Klintworth GK, Eds. 2nd Edition Marcel Dekker, NY, pp 1625-1710 (1994)). In the case of solid tumors, the neovascularization allows the tumor cells to acquire a growth advantage and proliferative autonomy compared to the normal cells. Accordingly, a correlation has been observed between density of microvessels in tumor sections and patient survival in breast cancer as well as in several  
15 other tumors (Weidner *et al. N Engl J Med* 324:1-6 (1991); Horak *et al. Lancet* 340:1120-1124 (1992); and Macchiarini *et al. Lancet* 340:145-146 (1992)).

The search for positive regulators of angiogenesis has yielded many candidates, including aFGF, bFGF, TGF- $\alpha$ , TGF- $\beta$ , HGF, TNF- $\alpha$ , angiogenin, IL-8, etc. (Folkman *et al.* and Klagsbrun *et al.*). The negative regulators so far identified include thrombospondin  
20 (Good *et al. Proc. Natl. Acad. Sci. USA.* 87:6624-6628 (1990)), the 16-kilodalton N-terminal fragment of prolactin (Clapp *et al. Endocrinology*, 133:1292-1299 (1993)), angiostatin (O'Reilly *et al. Cell*, 79:315-328 (1994)) and endostatin (O'Reilly *et al. Cell*, 88:277-285 (1996)).

Work done over the last several years has established the key role of vascular endothelial growth factor (VEGF) in the regulation of normal and abnormal angiogenesis (Ferrara *et al. Endocr. Rev.* 18:4-25 (1997)). The finding that the loss of even a single VEGF allele results in embryonic lethality points to an irreplaceable role played by this factor in the development and differentiation of the vascular system (Ferrara *et al.*). Furthermore, VEGF has been shown to be a key mediator of neovascularization associated with tumors and intraocular disorders (Ferrara *et al.*). The VEGF mRNA is overexpressed by the majority of human tumors examined (Berkman *et al. J Clin Invest* 91:153-159 (1993); Brown *et al. Human Pathol.* 26:86-91 (1995); Brown *et al. Cancer Res.* 53:4727-4735 (1993); Mattern *et al. Brit. J. Cancer.* 73:931-934 (1996); and Dvorak *et al. Am J. Pathol.* 146:1029-1039 (1995)). Also, the concentration of VEGF in eye fluids are highly correlated to the presence of active proliferation of blood vessels in patients with diabetic and other ischemia-related retinopathies (Aiello *et al. N. Engl. J. Med.* 331:1480-1487 (1994)). Furthermore, recent studies have demonstrated the localization of VEGF in choroidal neovascular membranes in patients affected by AMD (Lopez *et al. Invest. Ophthalmol. Vis. Sci.* 37:855-868 (1996)). Anti-VEGF neutralizing antibodies suppress the growth of a variety of human tumor cell lines in nude mice (Kim *et al. Nature* 362:841-844 (1993); Warren *et al. J. Clin. Invest.* 95:1789-1797 (1995); Borgström *et al. Cancer Res.* 56:4032-4039 (1996); and Melnyk *et al. Cancer Res.* 56:921-924 (1996)) and also inhibit intraocular angiogenesis in models of ischemic retinal disorders (Adamis *et al. Arch. Ophthalmol.* 114:66-71 (1996)). Therefore, anti-VEGF monoclonal antibodies or other inhibitors of VEGF action are promising candidates for the treatment of solid tumors and various intraocular neovascular disorders.

#### SUMMARY OF THE INVENTION

This application describes humanized anti-VEGF antibodies and anti-VEGF antibody variants with desirable properties from a therapeutic perspective, including strong binding affinity for VEGF; the ability to inhibit VEGF-induced proliferation of endothelial cells *in vitro*; and the ability to inhibit VEGF-induced angiogenesis *in vivo*.

The preferred humanized anti-VEGF antibody or variant anti-VEGF antibody herein binds human VEGF with a  $K_d$  value of no more than about  $1 \times 10^{-8}$ M and preferably no more than about  $5 \times 10^{-9}$ M. In addition, the humanized or variant anti-VEGF antibody may have an ED50 value of no more than about 5nM for inhibiting VEGF-induced proliferation of



endothelial cells *in vitro*. The humanized or variant anti-VEGF antibodies of particular interest herein are those which inhibit at least about 50% of tumor growth in an A673 *in vivo* tumor model, at an antibody dose of 5mg/kg.

In one embodiment, the anti-VEGF antibody has a heavy and light chain variable domain, wherein the heavy chain variable domain comprises hypervariable regions with the following amino acid sequences: CDRH1 (GYX<sub>1</sub>FTX<sub>2</sub>YGMN, wherein X<sub>1</sub> is T or D and X<sub>2</sub> is N or H; SEQ ID NO:128), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 (YPX<sub>1</sub>YYGX<sub>2</sub>SHWYFDV, wherein X<sub>1</sub> is Y or H and X<sub>2</sub> is S or T; SEQ ID NO:129). For example, the heavy chain variable domain may comprise the amino acid sequences of CDRH1 (GYTFTNYGMN; SEQ ID NO:1), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 (YPHYYGSSHWYFDV; SEQ ID NO:3). Preferably, the three heavy chain hypervariable regions are provided in a human framework region, *e.g.*, as a contiguous sequence represented by the following formula: FR1-CDRH1-FR2-CDRH2-FR3-CDRH3-FR4.

The invention further provides an anti-VEGF antibody heavy chain variable domain comprising the amino acid sequence:

EVQLVESGGGLVQPGGSLRLSCAASGYX<sub>1</sub>FTX<sub>2</sub>YGMNWVRQAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYPX<sub>3</sub>YYGX<sub>4</sub>SHWYFDVWGQGTLVTVSS (SEQ ID NO:125), wherein X<sub>1</sub> is T or D; X<sub>2</sub> is N or H; X<sub>3</sub> is Y or H and X<sub>4</sub> is S or T. One particularly useful heavy chain variable domain sequence is that of the F(ab)-12 humanized antibody of Example 1 and comprises the heavy chain variable domain sequence of SEQ ID NO:7. Such preferred heavy chain variable domain sequences may be combined with the following preferred light chain variable domain sequences or with other light chain variable domain sequences, provided that the antibody so produced binds human VEGF.

The invention also provides preferred light chain variable domain sequences which may be combined with the above-identified heavy chain variable domain sequences or with other heavy chain variable domain sequences, provided that the antibody so produced retains the ability to bind to human VEGF. For example, the light chain variable domain may comprise hypervariable regions with the following amino acid sequences: CDRL1 (SASQDISNYLN; SEQ ID NO:4), CDRL2 (FTSSLHS; SEQ ID NO:5) and CDRL3 (QQYSTVPWT; SEQ ID NO:6). Preferably, the three light chain hypervariable regions are

provided in a human framework region, *e.g.*, as a contiguous sequence represented by the following formula: FR1-CDRL1-FR2-CDRL2-FR3-CDRL3-FR4.

In one embodiment, the invention provides a humanized anti-VEGF antibody light chain variable domain comprising the amino acid sequence:

5 DIQX<sub>1</sub>TQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIIYFTSSLHS  
GVPSRFSGSGSGTDFTLTISLQPEDFATYYCQQYSTVPWTFGQGTKVEIKR (SEQ  
ID NO:124), wherein X<sub>1</sub> is M or L. One particularly useful light chain variable domain  
sequence is that of the F(ab)-12 humanized antibody of Example 1 and comprises the light  
chain variable domain sequence of SEQ ID NO:8.

10 The invention also provides a variant of a parent anti-VEGF antibody (which parent  
antibody is preferably a humanized or human anti-VEGF antibody), wherein the variant binds  
human VEGF and comprises an amino acid substitution in a hypervariable region of the heavy  
or light chain variable domain of the parent anti-VEGF antibody. The variant preferably has  
one or more substitution(s) in one or more hypervariable region(s) of the anti-VEGF  
15 antibody. Preferably, the substitution(s) are in the heavy chain variable domain of the parent  
antibody. For example, the amino acid substitution(s) may be in the CDRH1 and/or CDRH3  
of the heavy chain variable domain. Preferably, there are substitutions in both these  
hypervariable regions. Such "affinity matured" variants are demonstrated herein to bind  
human VEGF more strongly than the parent anti-VEGF antibody from which they are  
20 generated, *i.e.*, they have a K<sub>d</sub> value which is significantly less than that of the parent anti-  
VEGF antibody. Preferably, the variant has an ED50 value for inhibiting VEGF-induced  
proliferation of endothelial cells *in vitro* which is at least about 10 fold lower, preferably at  
least about 20 fold lower, and most preferably at least about 50 fold lower, than that of the  
parent anti-VEGF antibody. One particularly preferred variant is the Y0317 variant of  
25 Example 3, which has a CDRH1 comprising the amino acid sequence:GYDFTHYGMN  
(SEQ ID NO:126) and a CDRH3 comprising the amino acid  
sequence:YPYYYGTSHWYFDV (SEQ ID NO:127). These hypervariable regions and  
CDRH2 are generally provided in a human framework region, *e.g.*, resulting in a heavy chain  
variable domain comprising the amino acid sequence of SEQ ID NO:116. Such heavy chain  
30 variable domain sequences are optionally combined with a light chain variable domain  
comprising the amino acid sequence of SEQ ID NO:124, and preferably the light chain  
variable domain amino acid sequence of SEQ ID NO:115.

Various forms of the antibody are contemplated herein. For example, the anti-VEGF antibody may be a full length antibody (*e.g.* having an intact human Fc region) or an antibody fragment (*e.g.* a Fab, Fab' or F(ab')<sub>2</sub>). Furthermore, the antibody may be labeled with a detectable label, immobilized on a solid phase and/or conjugated with a heterologous compound (such as a cytotoxic agent).

Diagnostic and therapeutic uses for the antibody are contemplated. In one diagnostic application, the invention provides a method for determining the presence of VEGF protein comprising exposing a sample suspected of containing the VEGF protein to the anti-VEGF antibody and determining binding of the antibody to the sample. For this use, the invention provides a kit comprising the antibody and instructions for using the antibody to detect the VEGF protein.

The invention further provides: isolated nucleic acid encoding the antibody; a vector comprising that nucleic acid, optionally operably linked to control sequences recognized by a host cell transformed with the vector; a host cell comprising that vector; a process for producing the antibody comprising culturing the host cell so that the nucleic acid is expressed and, optionally, recovering the antibody from the host cell culture (*e.g.* from the host cell culture medium). The invention also provides a composition comprising the anti-VEGF antibody and a pharmaceutically acceptable carrier or diluent. The composition for therapeutic use is sterile and may be lyophilized. The invention further provides a method for treating a mammal suffering from a tumor or retinal disorder, comprising administering a therapeutically effective amount of the anti-VEGF antibody to the mammal.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B depict the amino acid sequences of variable heavy domain (SEQ ID NO:9) and light domain (SEQ ID NO:10) of muMAbVEGF A.4.6.1, variable heavy domain (SEQ ID NO:7) and light domain (SEQ ID NO:8) of humanized F(ab) (F(ab)-12) and human consensus frameworks (hum III for heavy subgroup III (SEQ ID NO:11); humκ1 for light κ subgroup I (SEQ ID NO:12)). Fig. 1A aligns variable heavy domain sequences and Fig. 1B aligns variable light domain sequences. Asterisks indicate differences between humanized F(ab)-12 and the murine MAb or between F(ab)-12 and the human framework. Complementarity Determining Regions (CDRs) are underlined.

Fig. 2 is a ribbon diagram of the model of humanized F(ab)-12 VL and VH domains. VL domain is shown in brown with CDRs in tan. The sidechain of residue L46 is shown in yellow. VH domain is shown in purple with CDRs in pink. Sidechains of VH residues changed from human to murine are shown in yellow.

5 Fig. 3 depicts inhibition of VEGF-induced mitogenesis by humanized anti-VEGF F(ab)-12 from Example 1. Bovine adrenal cortex-derived capillary endothelial cells were seeded at the density of  $6 \times 10^3$  cells/well in six well plates, as described in Example 1. Either muMAb VEGF A.4.6.1 or rhuMAb VEGF (IgG1; F(ab)-12) was added at the indicated concentrations. After 2-3 hours, rhVEGF165 was added at the final concentration of 3 ng/ml.  
10 After five or six days, cells were trypsinized and counted. Values shown are means of duplicate determinations. The variation from the mean did not exceed 10%.

Fig. 4 shows inhibition of tumor growth *in vivo* by humanized anti-VEGF F(ab)-12 from Example 1. A673 rhabdomyosarcoma cells were injected in BALB/c nude mice at the density of  $2 \times 10^6$  per mouse. Starting 24 hours after tumor cell inoculation, animals were  
15 injected with a control MAb, muMAb VEGF A4.6.1 or rhuVEGF MAb (IgG1; F(ab)-12) twice weekly, intra peritoneally. The dose of the control Mab was 5 mg/kg; the anti-VEGF MAbs were given at 0.5 or 5 mg/kg, as indicated (n = 10). Four weeks after tumor cell injection, animals were euthanized and tumors were removed and weighed. \*: significant difference when compared to the control group by ANOVA (p < 0.05).

20 Figs. 5A and 5B show the acid sequences of the light and heavy variable domains respectively of murine antibody A4.6.1 (SEQ ID NO:10 for the VL and SEQ ID NO:9 for the VH) and humanized A4.6.1 variants hu2.0 (SEQ ID NO:13 for the VL and SEQ ID NO:14 for the VH) and hu2.10 (SEQ ID NO:15 for the VL and SEQ ID NO:16 for the VH) from Example 2. Sequence numbering is according to Kabat *et al.*, *Sequences of Proteins of*  
25 *Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991) and mismatches are indicated by asterisks (murine A4.6.1 vs hu2.0) or bullets (hu2.0 vs hu2.10). Variant hu2.0 contains only the CDR sequences (bold) from the murine antibody grafted onto a human light chain  $\kappa$  subgroup I consensus framework (SEQ ID NO:12) and heavy chain subgroup III consensus framework (SEQ ID NO:11). hu2.10  
30 was the consensus humanized clone obtained from phage sorting experiments described herein.

Fig. 6 depicts framework residues targeted for randomization in Example 2.

Fig. 7 depicts the phagemid construct for surface display of Fab-pIII fusions on phage. The phagemid encodes a humanized version of the Fab fragment for antibody A4.6.1 fused to a portion of the M13 gene III coat protein. The fusion protein consists of the Fab joined at the carboxyl terminus of the heavy chain to a single glutamine residue (from suppression of an amber codon in *supE E. coli*), then the C-terminal region of the gene III protein (residues 249-406). Transformation into F<sup>+</sup> *E. coli*, followed by superinfection with M13KO7 helper phage, produces phagemid particles in which a small proportion of these display a single copy of the fusion protein.

Figs. 8A-E depict the double stranded nucleotide sequence (SEQ ID NO:99) for phage-display antibody vector phMB4-19-1.6 in Example 3 and the amino acid sequence encoded thereby (SEQ ID NO:100).

Figs. 9A and 9B depict an alignment of the amino acid sequences for the light and heavy variable domains respectively of affinity matured anti-VEGF variants in Example 3, compared to F(ab)-12 of Example 1 (SEQ ID NO's 8 and 7 for light and heavy variable domains, respectively). CDRs are underlined and designated by L, light, or H, heavy chain, and numbers 1-3. Residues are numbered sequentially in the VL and VH domains, as opposed to the Kabat numbering scheme. The template molecule, MB1.6 (SEQ ID NO's 101 and 102 for light and heavy variable domains, respectively) is shown, along with variants: H2305.6 (SEQ ID NO's 103 and 104 for light and heavy variable domains, respectively), Y0101 (SEQ ID NO's 105 and 106 for light and heavy variable domains, respectively), and Y0192 (SEQ ID NO's 107 and 108 for light and heavy variable domains, respectively). Differences from F(ab)-12 are shown in shaded boxes.

Figs. 10A and 10B depict an alignment of the amino acid sequences for the light and heavy variable domains respectively of affinity matured anti-VEGF variants from Example 3 compared to F(ab)-12 of Example 1 (SEQ ID NO's 8 and 7 for light and heavy variable domains, respectively). CDRs are underlined and designated by L, light, or H, heavy chain, and numbers 1-3. The variants are designated Y0243-1 (SEQ ID NO's 109 and 110 for light and heavy variable domains, respectively), Y0238-3 (SEQ ID NO's 111 and 112 for light and heavy variable domains, respectively), Y0313-1 (SEQ ID NO's 113 and 114 for light and heavy variable domains, respectively), and Y0317 (SEQ ID NO's 115 and 116 for light and heavy variable domains, respectively). Differences from F(ab)-12 are shown in shaded boxes.

Fig. 11 depicts the results of the HuVEC activity assay in Example 3 for variants Y0238-3, Y0192 and Y0313-1 as well as full length F(ab)-12 from Example 1.

Fig. 12 depicts inhibition of VEGF-induced mitogenesis by full length F(ab)-12 from Example 1 (rhuMAb VEGF), a Fab fragment of F(ab)-12 from Example 1 (rhuFab VEGF),  
5 and a Fab fragment of affinity matured variant Y0317 from Example 3 (rhuFab VEGF (affinity matured)).

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

### **I. Definitions**

10 The term "human VEGF" as used herein refers to the 165-amino acid human vascular endothelial cell growth factor, and related 121-, 189-, and 206-amino acid vascular endothelial cell growth factors, as described by Leung *et al.*, *Science* 246:1306 (1989), and Houck *et al.*, *Mol. Endocrin.* 5:1806 (1991) together with the naturally occurring allelic and processed forms of those growth factors.

15 The present invention provides anti-VEGF antagonistic antibodies which are capable of inhibiting one or more of the biological activities of VEGF, for example, its mitogenic or angiogenic activity. Antagonists of VEGF act by interfering with the binding of VEGF to a cellular receptor, by incapacitating or killing cells which have been activated by VEGF, or by interfering with vascular endothelial cell activation after VEGF binding to a cellular  
20 receptor. All such points of intervention by a VEGF antagonist shall be considered equivalent for purposes of this invention.

The term "VEGF receptor" or "VEGFr" as used herein refers to a cellular receptor for VEGF, ordinarily a cell-surface receptor found on vascular endothelial cells, as well as variants thereof which retain the ability to bind hVEGF. One example of a VEGF receptor  
25 is the *fms*-like tyrosine kinase (*flt*), a transmembrane receptor in the tyrosine kinase family. DeVries *et al.*, *Science* 255:989 (1992); Shibuya *et al.*, *Oncogene* 5:519 (1990). The *flt* receptor comprises an extracellular domain, a transmembrane domain, and an intracellular domain with tyrosine kinase activity. The extracellular domain is involved in the binding of VEGF, whereas the intracellular domain is involved in signal transduction. Another example  
30 of a VEGF receptor is the *flk-1* receptor (also referred to as KDR). Matthews *et al.*, *Proc. Nat. Acad. Sci.* 88:9026 (1991); Terman *et al.*, *Oncogene* 6:1677 (1991); Terman *et al.*, *Biochem. Biophys. Res. Commun.* 187:1579 (1992). Binding of VEGF to the *flt* receptor

results in the formation of at least two high molecular weight complexes, having apparent molecular weight of 205,000 and 300,000 Daltons. The 300,000 Dalton complex is believed to be a dimer comprising two receptor molecules bound to a single molecule of VEGF.

The term "epitope A4.6.1" when used herein, unless indicated otherwise, refers to the region of human VEGF to which the A4.6.1 antibody disclosed in Kim *et al.*, *Growth Factors* 7:53 (1992) and Kim *et al.* *Nature* 362:841 (1993), binds.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, *etc.* Preferably, the mammal is human.

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain ( $V_H$ ) followed by a number of constant domains. Each light chain has a variable domain at one end ( $V_L$ ) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three

segments called hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FRs (FR1, FR2, FR3 and FR4, respectively), largely adopting a  $\beta$ -sheet configuration, connected  
5 by three hypervariable regions, which form loops connecting, and in some cases forming part of, the  $\beta$ -sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of  
10 Health, Bethesda, MD. (1991), pages 647-669). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region  
15 comprises amino acid residues from a "complementarity determining region" or "CDR" (*i.e.* residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) and/or those residues from a "hypervariable  
20 loop" (*i.e.* residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

Papain digestion of antibodies produces two identical antigen-binding fragments,  
25 called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an  $F(ab')_2$  fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy chain and one  
30 light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the  $V_H$ - $V_L$  dimer. Collectively, the six hypervariable regions confer



antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first  
5 constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxyl terminus of the heavy chain CH1 domain including one or more cysteine(s) from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')<sub>2</sub> antibody fragments originally were produced as pairs of Fab' fragments which have  
10 hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa ( $\kappa$ ) and lambda ( $\lambda$ ), based on the amino acid sequences of their constant domains.

15 Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.*, IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called  $\alpha$ ,  
20  $\delta$ ,  $\epsilon$ ,  $\gamma$ , and  $\mu$ , respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antibody" herein is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), and antibody fragments so long as they  
25 exhibit the desired biological activity.

"Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable domain thereof. Examples of antibody fragments include Fab, Fab', F(ab')<sub>2</sub>, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

30 The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that

may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant  
5 on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al.*, *Nature* 256:495 (1975), or may be  
10 made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al.*, *Nature* 352:624-628 (1991) and Marks *et al.*, *J. Mol. Biol.* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies  
15 (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such  
20 antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; and Morrison *et al.*, *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

"Humanized" forms of non-human (*e.g.*, murine) antibodies are chimeric antibodies which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which  
25 hypervariable region residues of the recipient are replaced by hypervariable region residues from a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in  
30 the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the

hypervariable regions correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al.*,  
5 *Nature* 321:522-525 (1986); Reichmann *et al.*, *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

"Single-chain Fv" or "sFv" antibody fragments comprise the V<sub>H</sub> and V<sub>L</sub> domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V<sub>H</sub> and V<sub>L</sub> domains which  
10 enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V<sub>H</sub>) connected to a light chain variable domain (V<sub>L</sub>) in the same polypeptide chain (V<sub>H</sub> - V<sub>L</sub>). By using a linker that  
15 is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA* 90:6444-6448 (1993).

20 The expression "linear antibodies" when used throughout this application refers to the antibodies described in Zapata *et al. Protein Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V<sub>H</sub>-C<sub>H</sub>1-V<sub>H</sub>-C<sub>H</sub>1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

A "variant" anti-VEGF antibody, refers herein to a molecule which differs in amino  
25 acid sequence from a "parent" anti-VEGF antibody amino acid sequence by virtue of addition, deletion and/or substitution of one or more amino acid residue(s) in the parent antibody sequence. In the preferred embodiment, the variant comprises one or more amino acid substitution(s) in one or more hypervariable region(s) of the parent antibody. For example, the variant may comprise at least one, *e.g.* from about one to about ten, and  
30 preferably from about two to about five, substitutions in one or more hypervariable regions of the parent antibody. Ordinarily, the variant will have an amino acid sequence having at least 75% amino acid sequence identity with the parent antibody heavy or light chain variable

domain sequences (*e.g.* as in SEQ ID NO:7 or 8), more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, and most preferably at least 95%. Identity or homology with respect to this sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the parent antibody residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the antibody sequence shall be construed as affecting sequence identity or homology. The variant retains the ability to bind human VEGF and preferably has properties which are superior to those of the parent antibody. For example, the variant may have a stronger binding affinity, enhanced ability to inhibit VEGF-induced proliferation of endothelial cells and/or increased ability to inhibit VEGF-induced angiogenesis *in vivo*. To analyze such properties, one should compare a Fab form of the variant to a Fab form of the parent antibody or a full length form of the variant to a full length form of the parent antibody, for example, since it has been found that the format of the anti-VEGF antibody impacts its activity in the biological activity assays disclosed herein. The variant antibody of particular interest herein is one which displays at least about 10 fold, preferably at least about 20 fold, and most preferably at least about 50 fold, enhancement in biological activity when compared to the parent antibody.

The "parent" antibody herein is one which is encoded by an amino acid sequence used for the preparation of the variant. Preferably, the parent antibody has a human framework region and, if present, has human antibody constant region(s). For example, the parent antibody may be a humanized or human antibody.

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody *in situ* within recombinant

cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The term "epitope tagged" when used herein refers to the anti-VEGF antibody fused to an "epitope tag". The epitope tag polypeptide has enough residues to provide an epitope  
5 against which an antibody thereagainst can be made, yet is short enough such that it does not interfere with activity of the VEGF antibody. The epitope tag preferably is sufficiently unique so that the antibody thereagainst does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least 6 amino acid residues and usually between about 8-50 amino acid residues (preferably between about 9-30 residues). Examples include  
10 the flu HA tag polypeptide and its antibody 12CA5 (Field *et al. Mol. Cell. Biol.* 8:2159-2165 (1988)); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan *et al., Mol. Cell. Biol.* 5(12):3610-3616 (1985)); and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody (Paborsky *et al., Protein Engineering* 3(6):547-553 (1990)). In certain embodiments, the epitope tag is a "salvage receptor binding epitope". As used  
15 herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (*e.g.*, IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, or IgG<sub>4</sub>) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to  
20 include radioactive isotopes (*e.g.*, I<sup>131</sup>, I<sup>125</sup>, Y<sup>90</sup> and Re<sup>186</sup>), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include Adriamycin, Doxorubicin, 5-  
25 Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiotepa, Taxotere (docetaxel), Busulfan, Cytosine, Taxol, Methotrexate, Cisplatin, Melphalan, Vinblastine, Bleomycin, Etoposide, Ifosfamide, Mitomycin C, Mitoxantrone, Vincristine, Vinorelbine, Carboplatin, Teniposide, Daunomycin, Carminomycin, Aminopterin, Dactinomycin, Mitomycins, Esperamicins (see U.S. Pat. No. 4,675,187), Melphalan and other related  
30 nitrogen mustards.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared

to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, *e.g.*, Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella *et al.*, "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt *et al.*, (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs,  $\beta$ -lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the antibody. The label may itself be detectable by itself (*e.g.*, radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

By "solid phase" is meant a non-aqueous matrix to which the antibody of the present invention can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (*e.g.* controlled pore glass), polysaccharides (*e.g.*, agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (*e.g.* an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Patent No. 4,275,149.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-VEGF antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes. An "isolated" nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the antibody nucleic acid. An

isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the antibody where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The expression "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

## **II. Modes for Carrying out the Invention**

The examples hereinbelow describe the production of humanized and variant anti-

VEGF antibodies with desirable properties from a therapeutic perspective including: (a) strong binding affinity for the VEGF antigen; (b) an ability to inhibit VEGF-induced proliferation of endothelial cells *in vitro*; and (c) the ability to inhibit VEGF-induced angiogenesis *in vivo*.

5       Antibody affinities may be determined as described in the examples hereinbelow. Preferred humanized or variant antibodies are those which bind human VEGF with a  $K_d$  value of no more than about  $1 \times 10^{-7}M$ ; preferably no more than about  $1 \times 10^{-8}M$ ; and most preferably no more than about  $5 \times 10^{-9}M$ .

10       Aside from antibodies with strong binding affinity for human VEGF, it is also desirable to select humanized or variant antibodies which have other beneficial properties from a therapeutic perspective. For example, the antibody may be one which inhibits endothelial cell growth in response to VEGF. In one embodiment, the antibody may be able to inhibit bovine capillary endothelial cell proliferation in response to a near maximally effective concentration of VEGF (3 ng/ml). Preferably, the antibody has an effective dose  
15       50 (ED50) value of no more than about 5nM, preferably no more than about 1nM, and most preferably no more than about 0.5nM, for inhibiting VEGF-induced proliferation of endothelial cells in this "endothelial cell growth assay", *i.e.*, at these concentrations the antibody is able to inhibit VEGF-induced endothelial cell growth *in vitro* by 50%. A preferred "endothelial cell growth assay" involves culturing bovine adrenal cortex-derived  
20       capillary endothelial cells in the presence of low glucose Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% calf serum, 2 mM glutamine, and antibiotics (growth medium), essentially as described in Example 1 below. These endothelial cells are seeded at a density of  $6 \times 10^3$  cells per well, in 6-well plates in growth medium. Either parent anti-VEGF antibody (control), humanized or variant anti-VEGF antibody is then added at  
25       concentrations ranging between 1 and 5000 ng/ml. After 2-3 hr, purified VEGF was added to a final concentration of 3 ng/ml. For specificity control, each antibody may be added to endothelial cells at the concentration of 5000 ng/ml, either alone or in the presence of 2 ng/ml bFGF. After five or six days, cells are dissociated by exposure to trypsin and counted in a Coulter counter (Coulter Electronics, Hialeah, FL). Data may be analyzed by a four-  
30       parameter curve fitting program (KaleidaGraph).

      The preferred humanized or variant anti-VEGF antibody may also be one which has *in vivo* tumor suppression activity. For example, the antibody may suppress the growth of



human A673 rhabdomyosarcoma cells or breast carcinoma MDA-MB-435 cells in nude mice. For *in vivo* tumor studies, human A673 rhabdomyosarcoma cells (ATCC; CRL 1598) or MDA-MB-435 cells (available from the ATCC) are cultured in DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics as described in Example 1  
5 below. Female BALB/c nude mice, 6-10 weeks old, are injected subcutaneously with  $2 \times 10^6$  tumor cells in the dorsal area in a volume of 200  $\mu$ l. Animals are then treated with the humanized or variant antibody and a control antibody with no activity in this assay. The humanized or variant anti-VEGF MAb is administered at a dose of 0.5 and/or 5 mg/kg. Each MAb is administered twice weekly intra peritoneally in a volume of 100  $\mu$ l, starting 24 hr  
10 after tumor cell inoculation. Tumor size is determined at weekly intervals. Four weeks after tumor cell inoculation, animals are euthanized and the tumors are removed and weighed. Statistical analysis may be performed by ANOVA. Preferably, the antibody in this "*in vivo* tumor assay" inhibits about 50-100%, preferably about 70-100% and most preferably about 80-100% human A673 tumor cell growth at a dose of 5mg/kg.

15 In the preferred embodiment, the humanized or variant antibody fails to elicit an immunogenic response upon administration of a therapeutically effective amount of the antibody to a human patient. If an immunogenic response is elicited, preferably the response will be such that the antibody still provides a therapeutic benefit to the patient treated therewith.

20 The humanized or variant antibody is also preferably one which is able to inhibit VEGF-induced angiogenesis in a human, *e.g.* to inhibit human tumor growth and/or inhibit intraocular angiogenesis in retinal disorders.

Preferred antibodies bind the "epitope A4.6.1" as herein defined. To screen for antibodies which bind to the epitope on human VEGF bound by an antibody of interest (*e.g.*,  
25 those which block binding of the A4.6.1 antibody to human VEGF), a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping, *e.g.* as described in Champe *et al.*, *J. Biol. Chem.* 270:1388-1394 (1995), can be performed to determine whether the antibody binds an epitope of interest.

30 The antibodies of the preferred embodiment herein have a heavy chain variable domain comprising an amino acid sequence represented by the formula: FR1-CDRH1-FR2-CDRH2-FR3-CDRH3-FR4, wherein "FR1-4" represent the four framework regions and

“CDRH1-3” represent the three hypervariable regions of an anti-VEGF antibody variable heavy domain. FR1-4 may be derived from a “consensus sequence” (*i.e.* the most common amino acids of a class, subclass or subgroup of heavy or light chains of human immunoglobulins) as in the examples below or may be derived from an individual human antibody framework region or from a combination of different framework region sequences.

5 Many human antibody framework region sequences are compiled in Kabat *et al.*, *supra*, for example. In one preferred embodiment, the variable heavy FR is provided by a consensus sequence of a human immunoglobulin subgroup as compiled by Kabat *et al.*, *supra*. Preferably, the human immunoglobulin subgroup is human heavy chains subgroup III (*e.g.* as in SEQ ID NO:11).

10

The human variable heavy FR sequence preferably has substitutions therein, *e.g.* wherein the human FR residue is replaced by a corresponding nonhuman residue (by “corresponding nonhuman residue” is meant the nonhuman residue with the same Kabat positional numbering as the human residue of interest when the human and nonhuman sequences are aligned), but replacement with the nonhuman residue is not necessary. For example, a replacement FR residue other than the corresponding nonhuman residue may be selected by phage display (see Example 2 below). Exemplary variable heavy FR residues which may be substituted include any one or more of FR residue numbers: 37H, 49H, 67H, 69H, 71H, 73H, 75H, 76H, 78H, 94H (Kabat residue numbering employed here). Preferably

15

20 at least two, or at least three, or at least four of these residues are substituted. A particularly preferred combination of FR substitutions is: 49H, 69H, 71H, 73H, 76H, 78H, and 94H.

With respect to the heavy chain hypervariable regions, these preferably have amino acid sequences as follows:

#### CDRH1

25 GYX<sub>1</sub>X<sub>2</sub>X<sub>3</sub>X<sub>4</sub>YGX<sub>5</sub>N (SEQ ID NO:117), wherein X<sub>1</sub> is D, T or E, but preferably is D or T; X<sub>2</sub> is F, W, or Y, but preferably is F; X<sub>3</sub> is T, Q, G or S, but preferably is T; X<sub>4</sub> is H or N; and X<sub>5</sub> is M or I, but preferably is M.

#### CDRH2

30 WINTX<sub>1</sub>TGEPTYAADFKR (SEQ ID NO:118), wherein X<sub>1</sub> is Y or W, but preferably is Y.

## CDRH3

YPX<sub>1</sub>YX<sub>2</sub>X<sub>3</sub>X<sub>4</sub>X<sub>5</sub>HWYFDV (SEQ ID NO:119), wherein X<sub>1</sub> is H or Y; X<sub>2</sub> is Y, R, K, I, T, E, or W, but preferably is Y; X<sub>3</sub> is G, N, A, D, Q, E, T, K, or S, but preferably is G; X<sub>4</sub> is S, T, K, Q, N, R, A, E, or G, but preferably is S or T; and X<sub>5</sub> is S or G, but preferably is S.

5

The heavy chain variable domain optionally comprises what has been designated "CDR7" herein within (*i.e.* forming part of) FR3 (see Figs. 9B and 10B), wherein CDR7 may have the following amino acid sequence:

## 10 CDR7

X<sub>1</sub>SX<sub>2</sub>DX<sub>3</sub>X<sub>4</sub>X<sub>5</sub>X<sub>6</sub>TX<sub>7</sub> (SEQ ID NO:120), wherein X<sub>1</sub> is F, I, V, L, or A, but preferably is F; X<sub>2</sub> is A, L, V, or I, but preferably is L; X<sub>3</sub> is T, V or K, but preferably is T; X<sub>4</sub> is S or W, but preferably is S; X<sub>5</sub> is S, or K, but preferably is K; X<sub>6</sub> is N, or S, but preferably is S; and X<sub>7</sub> is V, A, L or I, but preferably is A.

15

The antibodies of the preferred embodiment herein have a light chain variable domain comprising an amino acid sequence represented by the formula: FR1-CDRL1-FR2-CDRL2-FR3-CDRL3-FR4, wherein "FR1-4" represent the four framework regions and "CDRL1-3" represent the three hypervariable regions of an anti-VEGF antibody variable heavy domain.

20 FR1-4 may be derived from a "consensus sequence" (*i.e.* the most common amino acids of a class, subclass or subgroup of heavy or light chains of human immunoglobulins) as in the examples below or may be derived from an individual human antibody framework region or from a combination of different framework region sequences. In one preferred embodiment, the variable light FR is provided by a consensus sequence of a human immunoglobulin subgroup as compiled by Kabat *et al.*, *supra*. Preferably, the human immunoglobulin subgroup is human kappa light chains subgroup I (*e.g.* as in SEQ ID NO:12).

25 The human variable light FR sequence preferably has substitutions therein, *e.g.* wherein the human FR residue is replaced by a corresponding mouse residue, but replacement with the nonhuman residue is not necessary. For example, a replacement residue other than the corresponding nonhuman residue may be selected by phage display (see Example 2 below). Exemplary variable light FR residues which may be substituted include any one or more of FR residue numbers: 4L, 46L and 71L (Kabat residue numbering employed here).

Preferably only 46L is substituted. In another embodiment, both 4L and 46L are substituted.

With respect to the CDRs, these preferably have amino acid sequences as follows:

#### CDRL1

$X_1AX_2X_3X_4X_5$ SNYLN (SEQ ID NO:121), wherein  $X_1$  is R or S, but preferably is S;  $X_2$  is  
5 S or N, but preferably is S;  $X_3$  is Q or E, but preferably is Q;  $X_4$  is Q or D, but preferably is  
D; and  $X_5$  is I or L, but preferably is I.

#### CDRL2

FTSSLHS (SEQ ID NO:122).

10

#### CDRL3

QQYSX $_1$ X $_2$ PWT (SEQ ID NO:123), wherein  $X_1$  is T, A or N, but preferably is T; and  $X_2$   
is V or T, but preferably is V.

15 Preferred humanized anti-VEGF antibodies are those having the heavy and/or light  
variable domain sequences of F(ab)-12 in Example 1 and variants thereof such as affinity  
matured forms including variants Y0317, Y0313-1 and Y0238-3 in Example 3, with Y0317  
being the preferred variant. Methods for generating humanized anti-VEGF antibodies of  
interest herein are elaborated in more detail below.

#### 20 A. Antibody Preparation

Methods for humanizing nonhuman VEGF antibodies and generating variants of anti-  
VEGF antibodies are described in the examples below. In order to humanize an anti-VEGF  
antibody, the nonhuman antibody starting material is prepared. Where a variant is to be  
generated, the parent antibody is prepared. Exemplary techniques for generating such  
25 nonhuman antibody starting material and parent antibodies will be described in the following  
sections.

##### (i) Antigen preparation

The VEGF antigen to be used for production of antibodies may be, *e.g.*, intact VEGF  
or a fragment of VEGF (*e.g.* a VEGF fragment comprising "epitope A4.6.1"). Other forms  
30 of VEGF useful for generating antibodies will be apparent to those skilled in the art. The  
VEGF antigen used to generate the antibody, is preferably human VEGF, *e.g.* as described  
in Leung *et al.*, *Science* 246:1306 (1989), and Houck *et al.*, *Mol. Endocrin.* 5:1806 (1991).

(ii) *Polyclonal antibodies*

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride,  $\text{SOCl}_2$ , or  $\text{R}^1\text{N}=\text{C}=\text{NR}$ , where R and  $\text{R}^1$  are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, *e.g.*, 100  $\mu\text{g}$  or 5  $\mu\text{g}$  of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(iii) *Monoclonal antibodies*

Monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al.*, *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium  
5 for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine  
10 myeloma lines, such as those derived from MOP-21 and M.C.-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*,  
15 133:3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation  
20 or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson *et al.*, *Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity,  
25 affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

30 The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel

electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Recombinant production of antibodies will be described in more detail below.

(iv) *Humanization and amino acid sequence variants*

Examples 1-2 below describe procedures for humanization of an anti-VEGF antibody. In certain embodiments, it may be desirable to generate amino acid sequence variants of these humanized antibodies, particularly where these improve the binding affinity or other biological properties of the humanized antibody. Example 3 describes methodologies for generating amino acid sequence variants of an anti-VEGF antibody with enhanced affinity relative to the parent antibody.

Amino acid sequence variants of the anti-VEGF antibody are prepared by introducing appropriate nucleotide changes into the anti-VEGF antibody DNA, or by peptide synthesis. Such variants include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the anti-VEGF antibodies of the examples herein. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the humanized or variant anti-VEGF antibody, such as changing the number or position of glycosylation sites.

A useful method for identification of certain residues or regions of the anti-VEGF antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis," as described by Cunningham and Wells *Science*, 244:1081-1085 (1989). Here, a residue or group of target residues are identified (*e.g.*, charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with VEGF antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined

by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed anti-VEGF antibody variants are screened for the desired activity. Alanine scanning mutagenesis is described in Example 3.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an anti-VEGF antibody with an N-terminal methionyl residue or the antibody fused to an epitope tag. Other insertional variants of the anti-VEGF antibody molecule include the fusion to the N- or C-terminus of the anti-VEGF antibody of an enzyme or a polypeptide which increases the serum half-life of the antibody (see below).

Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the anti-VEGF antibody molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in Table 1 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table 1, or as further described below in reference to amino acid classes, may be introduced and the products screened.

Table 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; asp, lys; arg	gln
Asp (D)	glu; asn	glu
Cys (C)	ser; ala	ser
Gln (Q)	asn; glu	asn
Glu (E)	asp; gln	asp



Original Residue	Exemplary Substitutions	Preferred Substitutions
Gly (G)	ala	ala
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	tyr
Pro (P)	ala	ala
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr; phe	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

- (1) hydrophobic: norleucine, met, ala, val, leu, ile;
- (2) neutral hydrophilic: cys, ser, thr;
- (3) acidic: asp, glu;
- (4) basic: asn, gln, his, lys, arg;
- (5) residues that influence chain orientation: gly, pro; and
- (6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

Any cysteine residue not involved in maintaining the proper conformation of the humanized or variant anti-VEGF antibody also may be substituted, generally with serine, to  
5 improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (*e.g.* a humanized or human  
10 antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants is affinity maturation using phage display (see Example 3 herein). Briefly, several hypervariable region sites (*e.g.* 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody  
15 variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (*e.g.* binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis (see Example 3) can be performed to identified hypervariable  
20 region residues contributing significantly to antigen binding. Alternatively, or in addition, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and human VEGF. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as  
25 described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Another type of amino acid variant of the antibody alters the original glycosylation pattern of the antibody. By altering is meant deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in  
30 the antibody.

Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The

tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked  
5 glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described  
10 tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

Nucleic acid molecules encoding amino acid sequence variants of the anti-VEGF antibody are prepared by a variety of methods known in the art. These methods include, but  
15 are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the anti-VEGF antibody.

(v) *Human antibodies*

20 As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region ( $J_H$ ) gene in chimeric and  
25 germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, *e.g.*, Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits *et al.*, *Nature*, 362:255-258 (1993); Bruggermann *et al.*, *Year in Immuno.*, 7:33 (1993); and US Patents  
30 5,591,669, 5,589,369 and 5,545,807. Human antibodies can also be derived from phage-display libraries (Hoogenboom *et al.*, *J. Mol. Biol.*, 227:381 (1991); Marks *et al.*, *J. Mol. Biol.*, 222:581-597 (1991); and US Patents 5,565,332 and 5,573,905). As discussed above,

human antibodies may also be generated by *in vitro* activated B cells (see US Patents 5,567,610 and 5,229,275)

(vi) *Antibody fragments*

In certain embodiments, the humanized or variant anti-VEGF antibody is an antibody  
5 fragment. Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, *e.g.*, Morimoto *et al.*, *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan *et al.*, *Science* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, Fab'-SH fragments can be directly recovered  
10 from *E. coli* and chemically coupled to form F(ab')<sub>2</sub> fragments (Carter *et al.*, *Bio/Technology* 10:163-167 (1992)). In another embodiment, the F(ab')<sub>2</sub> is formed using the leucine zipper GCN4 to promote assembly of the F(ab')<sub>2</sub> molecule. According to another approach, Fv, Fab or F(ab')<sub>2</sub> fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled  
15 practitioner.

(vii) *Multispecific antibodies*

In some embodiments, it may be desirable to generate multispecific (*e.g.* bispecific) humanized or variant anti-VEGF antibodies having binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the  
20 VEGF protein. Alternatively, an anti-VEGF arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (*e.g.*, CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the VEGF-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express VEGF. These  
25 antibodies possess an VEGF-binding arm and an arm which binds the cytotoxic agent (*e.g.*, saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (*e.g.*, F(ab')<sub>2</sub> bispecific antibodies).

According to another approach for making bispecific antibodies, the interface  
30 between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C<sub>H</sub>3 domain of an antibody constant domain. In this method,

one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (*e.g.*, tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (*e.g.*, alanine  
5 or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers. See WO96/27011 published September 6, 1996.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to  
10 biotin. Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using  
15 chemical linkage. Brennan *et al.*, *Science* 229:81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate F(ab')<sub>2</sub> fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is  
20 then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes. In yet a further embodiment, Fab'-SH fragments directly recovered from *E. coli* can be chemically coupled *in vitro* to form bispecific antibodies. Shalaby *et al.*, *J. Exp. Med.*  
25 175:217-225 (1992).

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al.*, *J. Immunol.* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab'  
30 portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers.

The "diabody" technology described by Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain ( $V_H$ ) connected to a light-chain variable domain ( $V_L$ ) by a linker which is too short to allow pairing between the two domains  
5 on the same chain. Accordingly, the  $V_H$  and  $V_L$  domains of one fragment are forced to pair with the complementary  $V_L$  and  $V_H$  domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al.*, *J. Immunol.* 152:5368 (1994). Alternatively, the bispecific antibody may be a "linear antibody" produced  
10 as described in Zapata *et al.* *Protein Eng.* 8(10):1057-1062 (1995).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al.*, *J. Immunol.* 147:60 (1991).

(viii) *Other modifications*

Other modifications of the humanized or variant anti-VEGF antibody are  
15 contemplated. For example, it may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased  
20 complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron *et al.*, *J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff *et al.*, *Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and  
25 may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson *et al.*, *Anti-Cancer Drug Design* 3:219-230 (1989).

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (*e.g.*, an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof),  
30 or a radioactive isotope (*i.e.*, a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used

include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolacca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-VEGF antibodies. Examples include  $^{212}\text{Bi}$ ,  $^{131}\text{I}$ ,  $^{131}\text{In}$ ,  $^{90}\text{Y}$  and  $^{186}\text{Re}$ .

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al.*, *Science* 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

In another embodiment, the antibody may be conjugated to a "receptor" (such as streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g., avidin) which is conjugated to a cytotoxic agent (e.g., a radionuclide).

The anti-VEGF antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein *et al.*, *Proc. Natl. Acad. Sci. USA* 82:3688 (1985); Hwang *et al.*, *Proc. Natl. Acad. Sci. USA* 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of

defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin *et al.*, *J. Biol. Chem.* 257:286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent (such as Doxorubicin) is optionally contained within the liposome.

- 5 See Gabizon *et al.*, *J. National Cancer Inst.* 81(19):1484 (1989)

The antibody of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (*e.g.*, a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Patent No. 4,975,278.

- 10 The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to convert it into its more active, cytotoxic form.

- Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as  $\beta$ -galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs;  $\beta$ -lactamase useful for converting drugs derivatized with  $\beta$ -lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, *e.g.*, Massey, *Nature* 328:457-458 (1987)). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

- The enzymes of this invention can be covalently bound to the anti-VEGF antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion



of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, *e.g.*, Neuberger *et al.*, *Nature* 312:604-608 (1984)).

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (*e.g.*, by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, *e.g.*, by DNA or peptide synthesis). See WO96/32478 published October 17, 1996.

The salvage receptor binding epitope generally constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (*e.g.*, of an IgG) and transferred to the CH1, CH3, or V<sub>H</sub> region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C<sub>L</sub> region or V<sub>L</sub> region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence: PKNSSMISNTP (SEQ ID NO:17), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:18), HQNLSDGK (SEQ ID NO:19), HQNISDGK (SEQ ID NO:20), or VISSHLGQ (SEQ ID NO:21), particularly where the antibody fragment is a Fab or F(ab')<sub>2</sub>. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s): HQNLSDGK (SEQ ID NO:19), HQNISDGK (SEQ ID NO:20), or VISSHLGQ (SEQ ID NO:21) and the sequence: PKNSSMISNTP (SEQ ID NO:17).

Covalent modifications of the humanized or variant anti-VEGF antibody are also included within the scope of this invention. They may be made by chemical synthesis or by enzymatic or chemical cleavage of the antibody, if applicable. Other types of covalent modifications of the antibody are introduced into the molecule by reacting targeted amino acid residues of the antibody with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues. Exemplary covalent modifications

of polypeptides are described in US Patent 5,534,615, specifically incorporated herein by reference. A preferred type of covalent modification of the antibody comprises linking the antibody to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos.  
5 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

#### **B. Vectors, Host Cells and Recombinant Methods**

The invention also provides isolated nucleic acid encoding the humanized or variant anti-VEGF antibody, vectors and host cells comprising the nucleic acid, and recombinant techniques for the production of the antibody.

10 For recombinant production of the antibody, the nucleic acid encoding it may be isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. In another embodiment, the antibody may be produced by homologous recombination, *e.g.* as described in US Patent 5,204,244, specifically incorporated herein by reference. DNA encoding the monoclonal antibody is readily isolated and sequenced using  
15 conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, *e.g.*, as described  
20 in US Patent 5,534,615 issued July 9, 1996 and specifically incorporated herein by reference.

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, *e.g.*, *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*,  
25 *Proteus*, *Salmonella*, *e.g.*, *Salmonella typhimurium*, *Serratia*, *e.g.*, *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (*e.g.*, *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC  
30 27,325) are suitable. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for anti-VEGF antibody-encoding vectors.

*Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces* hosts such as, e.g., *K. lactis*, *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickerhamii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilae* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus*; *Yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); *Candida*; *Trichoderma reesei* (EP 244,234); *Neurospora crassa*; *Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, e.g., *Neurospora*, *Penicillium*, *Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger*.

Suitable host cells for the expression of glycosylated anti-VEGF antibody are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, *J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub *et al.*, *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*,

*Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for anti-VEGF antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

The host cells used to produce the anti-VEGF antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham *et al.*, *Meth. Enz.* 58:44 (1979), Barnes *et al.*, *Anal. Biochem.* 102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter *et al.*, *Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a

commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

5           The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are  
10   based on human  $\gamma 1$ ,  $\gamma 2$ , or  $\gamma 4$  heavy chains (Lindmark *et al.*, *J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human  $\gamma 3$  (Guss *et al.*, *EMBO J.* 5:1567-1575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrene-divinyl)benzene allow for faster flow rates and shorter  
15   processing times than can be achieved with agarose. Where the antibody comprises a  $C_{H13}$  domain, the Bakerbond ABX<sup>TM</sup> resin (J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE<sup>TM</sup> chromatography on an anion or cation exchange resin (such as a  
20   polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed  
25   at low salt concentrations (*e.g.*, from about 0-0.25M salt).

### C.     Pharmaceutical Formulations

Therapeutic formulations of the antibody are prepared for storage by mixing the antibody having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed.  
30   (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids;

antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low  
5 molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming  
10 counter-ions such as sodium; metal complexes (*e.g.*, Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other (see Section F below). Such molecules are suitably  
15 present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacrylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin  
20 microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

25 Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, *e.g.*, films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat.  
30 No. 3,773,919), copolymers of L-glutamic acid and  $\gamma$  ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the Lupron Depot™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and

leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

#### **D. Non-therapeutic Uses for the Antibody**

The antibodies of the invention may be used as affinity purification agents. In this process, the antibodies are immobilized on a solid phase such as a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody is contacted with a sample containing the VEGF protein (or fragment thereof) to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the VEGF protein, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent, such as glycine buffer, pH 5.0, that will release the VEGF protein from the antibody.

Anti-VEGF antibodies may also be useful in diagnostic assays for VEGF protein, *e.g.*, detecting its expression in specific cells, tissues, or serum. Such diagnostic methods may be useful in cancer diagnosis.

For diagnostic applications, the antibody typically will be labeled with a detectable moiety. Numerous labels are available which can be generally grouped into the following categories:

(a) Radioisotopes, such as  $^{35}\text{S}$ ,  $^{14}\text{C}$ ,  $^{125}\text{I}$ ,  $^3\text{H}$ , and  $^{131}\text{I}$ . The antibody can be labeled with the radioisotope using the techniques described in *Current Protocols in Immunology*, Volumes 1 and 2, Coligen *et al.*, Ed. Wiley-Interscience, New York, New York, Pubs. (1991) for example and radioactivity can be measured using scintillation counting.

(b) Fluorescent labels such as rare earth chelates (europium chelates) or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, Lissamine, phycoerythrin and Texas

Red are available. The fluorescent labels can be conjugated to the antibody using the techniques disclosed in *Current Protocols in Immunology, supra*, for example. Fluorescence can be quantified using a fluorimeter.

(c) Various enzyme-substrate labels are available and U.S. Patent No. 4,275,149 provides a review of some of these. The enzyme generally catalyzes a chemical alteration of the chromogenic substrate which can be measured using various techniques. For example, the enzyme may catalyze a color change in a substrate, which can be measured spectrophotometrically. Alternatively, the enzyme may alter the fluorescence or chemiluminescence of the substrate. Techniques for quantifying a change in fluorescence are described above. The chemiluminescent substrate becomes electronically excited by a chemical reaction and may then emit light which can be measured (using a chemiluminometer, for example) or donates energy to a fluorescent acceptor. Examples of enzymatic labels include luciferases (*e.g.*, firefly luciferase and bacterial luciferase; U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, malate dehydrogenase, urease, peroxidase such as horseradish peroxidase (HRPO), alkaline phosphatase,  $\beta$ -galactosidase, glucoamylase, lysozyme, saccharide oxidases (*e.g.*, glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase), heterocyclic oxidases (such as uricase and xanthine oxidase), lactoperoxidase, microperoxidase, and the like. Techniques for conjugating enzymes to antibodies are described in O'Sullivan *et al.*, Methods for the Preparation of Enzyme-Antibody Conjugates for use in Enzyme Immunoassay, in *Methods in Enzym.* (ed J. Langone & H. Van Vunakis), Academic press, New York, 73:147-166 (1981).

Examples of enzyme-substrate combinations include, for example:

(i) Horseradish peroxidase (HRPO) with hydrogen peroxide as a substrate, wherein the hydrogen peroxidase oxidizes a dye precursor (*e.g.*, orthophenylene diamine (OPD) or 3,3',5,5'-tetramethyl benzidine hydrochloride (TMB));

(ii) alkaline phosphatase (AP) with para-Nitrophenyl phosphate as chromogenic substrate; and

(iii)  $\beta$ -D-galactosidase ( $\beta$ -D-Gal) with a chromogenic substrate (*e.g.*, p-nitrophenyl- $\beta$ -D-galactosidase) or fluorogenic substrate 4-methylumbelliferyl- $\beta$ -D-galactosidase.

Numerous other enzyme-substrate combinations are available to those skilled in the art. For a general review of these, see U.S. Patent Nos. 4,275,149 and 4,318,980.

Sometimes, the label is indirectly conjugated with the antibody. The skilled artisan



will be aware of various techniques for achieving this. For example, the antibody can be conjugated with biotin and any of the three broad categories of labels mentioned above can be conjugated with avidin, or *vice versa*. Biotin binds selectively to avidin and thus, the label can be conjugated with the antibody in this indirect manner. Alternatively, to achieve indirect  
5 conjugation of the label with the antibody, the antibody is conjugated with a small hapten (*e.g.*, digoxin) and one of the different types of labels mentioned above is conjugated with an anti-hapten antibody (*e.g.*, anti-digoxin antibody). Thus, indirect conjugation of the label with the antibody can be achieved.

In another embodiment of the invention, the anti-VEGF antibody need not be labeled,  
10 and the presence thereof can be detected using a labeled antibody which binds to the VEGF antibody.

The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, *Monoclonal Antibodies: A Manual of Techniques*,  
15 pp.147-158 (CRC Press, Inc. 1987).

Competitive binding assays rely on the ability of a labeled standard to compete with the test sample analyte for binding with a limited amount of antibody. The amount of VEGF protein in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes  
20 bound, the antibodies generally are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte which remain unbound.

Sandwich assays involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected. In a sandwich  
25 assay, the test sample analyte is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three-part complex. See, *e.g.*, US Pat No. 4,376,110. The second antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay).  
30 For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

For immunohistochemistry, the tumor sample may be fresh or frozen or may be embedded in paraffin and fixed with a preservative such as formalin, for example.

The antibodies may also be used for *in vivo* diagnostic assays. Generally, the antibody is labeled with a radio nuclide (such as <sup>111</sup>In, <sup>99</sup>Tc, <sup>14</sup>C, <sup>131</sup>I, <sup>125</sup>I, <sup>3</sup>H, <sup>32</sup>P or <sup>35</sup>S) so  
5 that the tumor can be localized using immunoscintigraphy.

#### **E. Diagnostic Kits**

As a matter of convenience, the antibody of the present invention can be provided in a kit, *i.e.*, a packaged combination of reagents in predetermined amounts with instructions for performing the diagnostic assay. Where the antibody is labeled with an enzyme, the kit  
10 will include substrates and cofactors required by the enzyme (*e.g.*, a substrate precursor which provides the detectable chromophore or fluorophore). In addition, other additives may be included such as stabilizers, buffers (*e.g.*, a block buffer or lysis buffer) and the like. The relative amounts of the various reagents may be varied widely to provide for concentrations in solution of the reagents which substantially optimize the sensitivity of the assay.  
15 Particularly, the reagents may be provided as dry powders, usually lyophilized, including excipients which on dissolution will provide a reagent solution having the appropriate concentration.

#### **F. Therapeutic Uses for the Antibody**

For therapeutic applications, the anti-VEGF antibodies of the invention are  
20 administered to a mammal, preferably a human, in a pharmaceutically acceptable dosage form such as those discussed above, including those that may be administered to a human intravenously as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intra-cerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. The antibodies also are suitably administered by intra  
25 tumoral, peritumoral, intralesional, or perilesional routes, to exert local as well as systemic therapeutic effects. The intraperitoneal route is expected to be particularly useful, for example, in the treatment of ovarian tumors.

For the prevention or treatment of disease, the appropriate dosage of antibody will depend on the type of disease to be treated, as defined above, the severity and course of the  
30 disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time

or over a series of treatments.

The anti-VEGF antibodies are useful in the treatment of various neoplastic and non-neoplastic diseases and disorders. Neoplasms and related conditions that are amenable to treatment include breast carcinomas, lung carcinomas, gastric carcinomas, esophageal carcinomas, colorectal carcinomas, liver carcinomas, ovarian carcinomas, thecomas, arrhenoblastomas, cervical carcinomas, endometrial carcinoma, endometrial hyperplasia, endometriosis, fibrosarcomas, choriocarcinoma, head and neck cancer, nasopharyngeal carcinoma, laryngeal carcinomas, hepatoblastoma, Kaposi's sarcoma, melanoma, skin carcinomas, hemangioma, cavernous hemangioma, hemangioblastoma, pancreas carcinomas, retinoblastoma, astrocytoma, glioblastoma, Schwannoma, oligodendroglioma, medulloblastoma, neuroblastomas, rhabdomyosarcoma, osteogenic sarcoma, leiomyosarcomas, urinary tract carcinomas, thyroid carcinomas, Wilm's tumor, renal cell carcinoma, prostate carcinoma, abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), and Meigs' syndrome.

Non-neoplastic conditions that are amenable to treatment include rheumatoid arthritis, psoriasis, atherosclerosis, diabetic and other proliferative retinopathies including retinopathy of prematurity, retrolental fibroplasia, neovascular glaucoma, age-related macular degeneration, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, chronic inflammation, lung inflammation, nephrotic syndrome, preeclampsia, ascites, pericardial effusion (such as that associated with pericarditis), and pleural effusion.

Age-related macular degeneration (AMD) is a leading cause of severe visual loss in the elderly population. The exudative form of AMD is characterized by choroidal neovascularization and retinal pigment epithelial cell detachment. Because choroidal neovascularization is associated with a dramatic worsening in prognosis, the VEGF antibodies of the present invention are expected to be especially useful in reducing the severity of AMD.

Depending on the type and severity of the disease, about 1  $\mu\text{g/kg}$  to about 50 mg/kg (e.g., 0.1-20mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily or weekly dosage might range from about 1  $\mu\text{g/kg}$  to about 20 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is repeated until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful.

The progress of this therapy is easily monitored by conventional techniques and assays, including, for example, radiographic tumor imaging.

According to another embodiment of the invention, the effectiveness of the antibody in preventing or treating disease may be improved by administering the antibody serially or  
5 in combination with another agent that is effective for those purposes, such as tumor necrosis factor (TNF), an antibody capable of inhibiting or neutralizing the angiogenic activity of acidic or basic fibroblast growth factor (FGF) or hepatocyte growth factor (HGF), an antibody capable of inhibiting or neutralizing the coagulant activities of tissue factor, protein C, or protein S (see Esmon *et al.*, PCT Patent Publication No. WO 91/01753, published 21  
10 February 1991), an antibody capable of binding to HER2 receptor (see Hudziak *et al.*, PCT Patent Publication No. WO 89/06692, published 27 July 1989), or one or more conventional therapeutic agents such as, for example, alkylating agents, folic acid antagonists, anti-metabolites of nucleic acid metabolism, antibiotics, pyrimidine analogs, 5-fluorouracil, cisplatin, purine nucleosides, amines, amino acids, triazol nucleosides, or corticosteroids.  
15 Such other agents may be present in the composition being administered or may be administered separately. Also, the antibody is suitably administered serially or in combination with radiological treatments, whether involving irradiation or administration of radioactive substances.

In one embodiment, vascularization of tumors is attacked in combination therapy.  
20 The antibody and one or more other anti-VEGF antagonists are administered to tumor-bearing patients at therapeutically effective doses as determined for example by observing necrosis of the tumor or its metastatic foci, if any. This therapy is continued until such time as no further beneficial effect is observed or clinical examination shows no trace of the tumor or any metastatic foci. Then TNF is administered, alone or in combination with an auxiliary  
25 agent such as alpha-, beta-, or gamma-interferon, anti-HER2 antibody, heregulin, anti-heregulin antibody, D-factor, interleukin-1 (IL-1), interleukin-2 (IL-2), granulocyte-macrophage colony stimulating factor (GM-CSF), or agents that promote microvascular coagulation in tumors, such as anti-protein C antibody, anti-protein S antibody, or C4b binding protein (see Esmon *et al.*, PCT Patent Publication No. WO 91/01753, published 21  
30 February 1991), or heat or radiation.

Since the auxiliary agents will vary in their effectiveness it is desirable to compare their impact on the tumor by matrix screening in conventional fashion. The administration

of anti-VEGF antibody and TNF is repeated until the desired clinical effect is achieved. Alternatively, the anti-VEGF antibody is administered together with TNF and, optionally, auxiliary agent(s). In instances where solid tumors are found in the limbs or in other locations susceptible to isolation from the general circulation, the therapeutic agents described herein are administered to the isolated tumor or organ. In other embodiments, a FGF or platelet-derived growth factor (PDGF) antagonist, such as an anti-FGF or an anti-PDGF neutralizing antibody, is administered to the patient in conjunction with the anti-VEGF antibody. Treatment with anti-VEGF antibodies optimally may be suspended during periods of wound healing or desirable neovascularization.

#### 10           **G.     Articles of Manufacture**

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container and a label. Suitable containers include, for example, bottles, vials, syringes, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The active agent in the composition is the anti-VEGF antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

25

#### EXAMPLE 1

This example describes the production of humanized anti-VEGF antibodies with desirable properties from a therapeutic standpoint.

#### **MATERIALS AND METHODS**

30           *Cloning of Murine A4.6.1 MAb and Construction of Mouse-Human Chimeric Fab:* The murine anti-VEGF mAb A4.6.1 has been previously described by Kim *et al.*, *Growth Factors* 7:53 (1992) and Kim *et al. Nature* 362:841 (1993). Total RNA was isolated

from hybridoma cells producing the anti-VEGF Mab A.4.6.1 using RNAsol (TEL-TEST) and reverse-transcribed to cDNA using Oligo-dT primer and the SuperScript II system (GIBCO BRL, Gaithersburg, MD). Degenerate oligonucleotide primer pools, based of the N-terminal amino acid sequences of the light and heavy chains of the antibody, were synthesized and used as forward primers. Reverse primers were based on framework 4 sequences obtained from murine light chain subgroup kV and heavy chain subgroup II (Kabat *et al. Sequences of Proteins of Immunological Interest*. 5th ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). After polymerase chain reaction (PCR) amplification, DNA fragments were ligated to a TA cloning vector (Invitrogen, San Diego, CA). Eight clones each of the light and heavy chains were sequenced. One clone with a consensus sequence for the light chain VL domain and one with a consensus sequence for the heavy chain VH domain were subcloned respectively into the pEMX1 vector containing the human CL and CH1 domains (Werther *et al. J. Immunol.* 157:4986-4995 (1996)), thus generating a mouse-human chimera. This chimeric F(ab) consisted of the entire murine A4.6.1 VH domain fused to a human CH1 domain at amino acid SerH113 and the entire murine A4.6.1 VL domain fused to a human CL domain at amino acid LysL107. Expression and purification of the chimeric F(ab) were identical to that of the humanized F(ab)s. The chimeric F(ab) was used as the standard in the binding assays.

**Computer Graphics Models of Murine and Humanized F(ab):** Sequences of the VL and VH domains (Figs. 1A and 1B) were used to construct a computer graphics model of the murine A4.6.1 VL-VH domains. This model was used to determine which framework residues should be incorporated into the humanized antibody. A model of the humanized F(ab) was also constructed to verify correct selection of murine framework residues. Construction of models was performed as described previously (Carter *et al. Proc. Natl. Acad. Sci. USA* 89:4285-4289 (1992) and Eigenbrot *et al. J.Mol. Biol.* 229:969-995 (1993)).

**Construction of Humanized F(ab)s:** The plasmid pEMX1 used for mutagenesis and expression of F(ab)s in *E. coli* has been described previously (Werther *et al., supra*). Briefly, the plasmid contains a DNA fragment encoding a consensus human k subgroup I light chain (VLkI-CL) and a consensus human subgroup III heavy chain (VHIII-CH1) and an alkaline phosphatase promoter. The use of the consensus sequences for VL and VH has been described previously (Carter *et al., supra*).

To construct the first F(ab) variant of humanized A4.6.1, F(ab)-1, site-directed mutagenesis (Kunkel *et al.*, *Proc. Natl. Acad. Sci. USA* 82:488-492 (1985)) was performed on a deoxyuridine-containing template of pEMX1. The six CDRs according to Kabat *et al.*, *supra*, were changed to the murine A4.6.1 sequence. F(ab)-1 therefore consisted of a complete human framework (VL k subgroup I and VH subgroup III) with the six complete murine CDR sequences. Plasmids for all other F(ab) variants were constructed from the plasmid template of F(ab)-1. Plasmids were transformed into *E. coli* strain XL-1 Blue (Stratagene, San Diego, CA) for preparation of double- and single-stranded DNA. For each variant, DNA coding for light and heavy chains was completely sequenced using the dideoxynucleotide method (Sequenase, U.S. Biochemical Corp., Cleveland, OH). Plasmids were transformed into *E. coli* strain 16C9, a derivative of MM294, plated onto Luria broth plates containing 50 µg/ml carbenicillin, and a single colony selected for protein expression. The single colony was grown in 5 ml Luria broth-100 mg/ml carbenicillin for 5-8 h at 37°C. The 5 ml culture was added to 500 ml AP5-50 µg/ml carbenicillin and allowed to grow for 20 h in a 4 L baffled shake flask at 30°C. AP5 media consists of: 1.5 g glucose, 11.0 g Hycase SF, 0.6 g yeast extract (certified), 0.19 g MgSO<sub>4</sub> (anhydrous), 1.07 g NH<sub>4</sub>Cl, 3.73 g KCl, 1.2 g NaCl, 120 ml 1 M triethanolamine, pH 7.4, to 1 L water and then sterile filtered through 0.1 mm Sealkeen filter. Cells were harvested by centrifugation in a 1 L centrifuge bottle at 3000xg and the supernatant removed. After freezing for 1 h, the pellet was resuspended in 25 ml cold 10 mM Tris-1 mM EDTA-20% sucrose, pH 8.0. 250 ml of 0.1 M benzamidine (Sigma, St. Louis, MO) was added to inhibit proteolysis. After gentle stirring on ice for 3 h, the sample was centrifuged at 40,000xg for 15 min. The supernatant was then applied to a protein G-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) column (0.5 ml bed volume) equilibrated with 10 mM Tris-1 mM EDTA, pH 7.5. The column was washed with 10 ml of 10 mM Tris-1 mM EDTA, pH 7.5, and eluted with 3 ml 0.3 M glycine, pH 3.0, into 1.25 ml 1 M Tris, pH 8.0. The F(ab) was then buffer exchanged into PBS using a Centricon-30 (Amicon, Beverly, MA) and concentrated to a final volume of 0.5 ml. SDS-PAGE gels of all F(ab)s were run to ascertain purity and the molecular weight of each variant was verified by electrospray mass spectrometry.

**Construction and Expression of Chimeric and Humanized IgG:** For generation of human IgG1 variants of chimeric (chIgG1) and humanized (huIgG1) A4.6.1, the appropriate murine or humanized VL and VH (F(ab)-12, Table 2) domains were subcloned

into separate, previously described, pRK vectors (Eaton *et al.*, *Biochemistry* 25:8343-8347 (1986)). The DNA coding for the entire light and the entire heavy chain of each variant was verified by dideoxynucleotide sequencing.

For transient expression of variants, heavy and light chain plasmids were co-  
5 transfected into human 293 cells (Graham *et al.*, *J. Gen. Virol.* 36:59-74 (1977)), using a high efficiency procedure (Gorman *et al.*, *DNA Prot. Eng. Tech.* 2:3-10 (1990)). Media was changed to serum-free and harvested daily for up to five days. Antibodies were purified from the pooled supernatants using protein A-Sepharose CL-4B (Pharmacia). The eluted antibody was buffer exchanged into PBS using a Centricon-30 (Amicon), concentrated to 0.5 ml,  
10 sterile filtered using a Millex-GV (Millipore, Bedford, MA) and stored at 4°C.

For stable expression of the final humanized IgG1 variant (rhuMAb VEGF), Chinese hamster ovary (CHO) cells were transfected with dicistronic vectors designed to coexpress both heavy and light chains (Lucas *et al.*, *Nucleic Acid Res.* 24:1774-79 (1996)). Plasmids were introduced into DP12 cells, a proprietary derivative of the CHO-K1 DUX B11 cell line  
15 developed by L. Chasin (Columbia University), via lipofection and selected for growth in GHT-free medium (Chisholm, V. High efficiency gene transfer in mammalian cells. In: Glover, DM, Hames, BD. *DNA Cloning 4. Mammalian systems*. Oxford Univ. Press, Oxford pp 1-41 (1996)). Approximately 20 unamplified clones were randomly chosen and reseeded into 96 well plates. Relative specific productivity of each colony was monitored using an  
20 ELISA to quantitate the full length human IgG accumulated in each well after 3 days and a fluorescent dye, Calcein AM, as a surrogate marker of viable cell number per well. Based on these data, several unamplified clones were chosen for further amplification in the presence of increasing concentrations of methotrexate. Individual clones surviving at 10, 50, and 100 nM methotrexate were chosen and transferred to 96 well plates for productivity screening.  
25 One clone, which reproducibly exhibited high specific productivity, was expanded in T-flasks and used to inoculate a spinner culture. After several passages, the suspension-adapted cells were used to inoculate production cultures in GHT-containing, serum-free media supplemented with various hormones and protein hydrolysates. Harvested cell culture fluid containing rhuMAb VEGF was purified using protein A-Sepharose CL-4B. The purity after  
30 this step was ~99%. Subsequent purification to homogeneity was carried out using an ion exchange chromatography step. The endotoxin content of the final purified antibody was < 0.10 eu/mg.



*F(ab) and IgG Quantitation:* For quantitating F(ab) molecules, ELISA plates were coated with 2 µg/ml goat anti-human IgG Fab (Organon Teknika, Durham, NC) in 50 mM carbonate buffer, pH 9.6, at 4°C overnight and blocked with PBS-0.5% bovine serum albumin (blocking buffer) at room temperature for 1 h. Standards (0.78 - 50 ng/ml human F(ab)) were purchased from Chemicon (Temecula, CA). Serial dilutions of samples in PBS-0.5% bovine serum albumin-0.05% polysorbate 20 (assay buffer) were incubated on the plates for 2 h. Bound F(ab) was detected using horseradish peroxidase-labeled goat anti-human IgG F(ab) (Organon Teknika), followed by 3,3',5,5'-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD) as the substrate. Plates were washed between steps. Absorbance was read at 450 nm on a Vmax plate reader (Molecular Devices, Menlo Park, CA). The standard curve was fit using a four-parameter nonlinear regression curve-fitting program. Data points which fell in the range of the standard curve were used for calculating the F(ab) concentrations of samples. The concentration of full-length antibody was determined using goat anti-human IgG Fc (Cappel, Westchester, PA) for capture and horseradish peroxidase-labeled goat anti-human Fc (Cappel) for detection. Human IgG1 (Chemicon) was used as standard.

*VEGF Binding Assay:* For measuring the VEGF binding activity of F(ab)s, ELISA plates were coated with 2 µg/ml rabbit F(ab')<sub>2</sub> to human IgG Fc (Jackson ImmunoResearch, West Grove, PA) and blocked with blocking buffer (described above). Diluted conditioned medium containing 3 ng/ml of KDR-IgG (Park *et al.*, *J. Biol. Chem.* 269:25646-25645 (1994)) in blocking buffer were incubated on the plate for 1 h. Standards (6.9 - 440 ng/ml chimeric F(ab)) and two-fold serial of samples were incubated with 2 nM biotinylated VEGF for 1 h in tubes. The solutions from the tubes were then transferred to the ELISA plates and incubated for 1 h. After washing, biotinylated VEGF bound to KDR was detected using horseradish peroxidase-labeled streptavidin (Zymed, South San Francisco, CA or Sigma, St. Louis, MO) followed by 3,3',5,5'-tetramethylbenzidine as the substrate. Titration curves were fit with a four-parameter nonlinear regression curve-fitting program (KaleidaGraph, Synergy Software, Reading PA). Concentrations of F(ab) variants corresponding to the midpoint absorbance of the titration curve of the standard were calculated and then divided by the concentration of the standard corresponding to the midpoint absorbance of the standard titration curve. Assays for full-length IgG were the same as for the F(ab)s except that the assay buffer contained 10% human serum.

**BIAcore™ Biosensor Assay:** VEGF binding of the humanized and chimeric F(ab)s were compared using a BIAcore™ biosensor (Karlsson *et al. Methods: A Comparison to Methods in Enzymology* 6:97-108 (1994)). Concentrations of F(ab)s were determined by quantitative amino acid analysis. VEGF was coupled to a CM-5 biosensor chip through  
5 primary amine groups according to manufacturer's instructions (Pharmacia). Off-rate kinetics were measured by saturating the chip with F(ab) (35  $\mu$ l of 2  $\mu$ M F(ab) at a flow rate of 20  $\mu$ l/min) and then switching to buffer (PBS-0.05% polysorbate 20). Data points from 0 - 4500 sec were used for off-rate kinetic analysis. The dissociation rate constant ( $k_{off}$ ) was obtained from the slope of the plot of  $\ln(R_0/R)$  versus time, where  $R_0$  is the signal at  $t=0$  and  
10  $R$  is the signal at each time point.

On-rate kinetics were measured using two-fold serial dilutions of F(ab) (0.0625 - 2 mM). The slope,  $K_s$ , was obtained from the plot of  $\ln(-dR/dt)$  versus time for each F(ab) concentration using the BIAcore™ kinetics evaluation software as described in the Pharmacia Biosensor manual.  $R$  is the signal at time  $t$ . Data between 80 and 168, 148, 128, 114, 102,  
15 and 92 sec were used for 0.0625, 0.125, 0.25, 0.5, 1, and 2 mM F(ab), respectively. The association rate constant ( $k_{on}$ ) was obtained from the slope of the plot of  $K_s$  versus F(ab) concentration. At the end of each cycle, bound F(ab) was removed by injecting 5  $\mu$ l of 50 mM HCl at a flow rate of 20  $\mu$ l/min to regenerate the chip.

**Endothelial Cell Growth Assay:** Bovine adrenal cortex-derived capillary endothelial  
20 cells were cultured in the presence of low glucose Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% calf serum, 2 mM glutamine, and antibiotics (growth medium), essentially as previously described (Leung *et al. Science* 246:1306-1309 (1989)). For mitogenic assays, endothelial cells were seeded at a density of  $6 \times 10^3$  cells per well, in 6-well plates in growth medium. Either muMAb VEGF A.4.6.1 or rhuMAb VEGF  
25 was then added at concentrations ranging between 1 and 5000 ng/ml. After 2-3 hr, purified *E.coli*-expressed rhVEGF165 was added to a final concentration of 3 ng/ml. For specificity control, each antibody was added to endothelial cells at the concentration of 5000 ng/ml, either alone or in the presence of 2 ng/ml bFGF. After five or six days, cells were dissociated by exposure to trypsin and counted in a Coulter counter (Coulter Electronics, Hialeah, FL).  
30 The variation from the mean did not exceed 10%. Data were analyzed by a four-parameter curve fitting program (KaleidaGraph).

*In Vivo Tumor Studies:* Human A673 rhabdomyosarcoma cells (ATCC; CRL 1598) were cultured as previously described in DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics (Kim *et al. Nature* 362:841-844 (1993) and Borgström *et al. Cancer Res.* 56:4032-4039 (1996)). Female BALB/c nude mice, 6-10 weeks old, were injected subcutaneously with  $2 \times 10^6$  tumor cells in the dorsal area in a volume of 200  $\mu$ l. Animals were then treated with muMAb VEGF A.4.6.1, rhuMAb VEGF or a control MAb directed against the gp120 protein (Kim *et al. Nature* 362:841-844 (1993)). Both anti-VEGF MAbs were administered at the doses of 0.5 and 5 mg/kg; the control MAb was given at the dose of 5 mg/kg. Each MAb was administered twice weekly intra peritoneally in a volume of 100  $\mu$ l, starting 24 hr after tumor cell inoculation. Each group consisted of 10 mice. Tumor size was determined at weekly intervals. Four weeks after tumor cell inoculation, animals were euthanized and the tumors were removed and weighed. Statistical analysis was performed by ANOVA.

15

## RESULTS

*Humanization:* The consensus sequence for the human heavy chain subgroup III and the light chain subgroup k I were used as the framework for the humanization (Kabat *et al., supra*) (Figs. 1A and 1B). This framework has been successfully used in the humanization of other murine antibodies (Werther *et al., supra*; Carter *et al., supra*; Presta *et al. J. Immunol.* 151:2623-2632 (1993); and Eigenbrot *et al. Proteins* 18:49-62 (1994)). CDR-H1 included residues H26-H35. The other CDRs were according to Kabat *et al., supra*. All humanized variants were initially made and screened for binding as F(ab)s expressed in *E. coli*. Typical yields from 500 ml shake flasks were 0.1-0.4 mg F(ab).

The chimeric F(ab) was used as the standard in the binding assays. In the initial variant, F(ab)-1, the CDR residues were transferred from the murine antibody to the human framework and, based on the models of the murine and humanized F(ab)s, the residue at position H49 (Ala in human) was changed to the murine Gly. In addition, F(ab)s which consisted of the chimeric heavy chain/F(ab)-1 light chain (F(ab)-2) and F(ab)-1 heavy chain/chimeric light chain (F(ab)-3) were generated and tested for binding. F(ab)-1 exhibited a binding affinity greater than 1000-fold reduced from the chimeric F(ab) (Table 2). Comparing the binding affinities of F(ab)-2 and F(ab)-3 suggested that framework residues in the F(ab)-1 VH domain needed to be altered in order to increase binding.

Table 2: Binding of Humanized Anti-VEGF F(ab) Variants to VEGF<sup>a</sup>

Variant	Template	Changes <sup>b</sup>	Purpose	EC50 F(ab)-X		
				EC50 chimeric F(ab) <sup>c</sup>		
				Mean	S.D.	N
chim-F(ab)	Chimeric F(ab)			1.0		
F(ab)-1	Human FR		Straight CDR swap AlaH49 <u>Gly</u>	>1350		2
5 F(ab)-2			Chimera Light Chain F(ab)-1 Heavy Chain	>145		3
F(ab)-3			F(ab)-1 Light Chain Chimera Heavy Chain	2.6	0.1	2
F(ab)-4	F(ab)-1	ArgH71 <u>Leu</u> AsnH73 <u>Thr</u>	CDR-H2 conformation Framework	>295		3
F(ab)-5	F(ab)-4	LeuL46 <u>Val</u>	VL-VH interface	80.9	6.5	2
F(ab)-6	F(ab)-5	LeuH78 <u>Ala</u>	CDR-H1 conformation	36.4	4.2	2
10 F(ab)-7	F(ab)-5	IleH69 <u>Phe</u>	CDR-H2 conformation	45.2	2.3	2
F(ab)-8	F(ab)-5	IleH69 <u>Phe</u> LeuH78 <u>Ala</u>	CDR-H2 conformation CDR-H1 conformation	9.6	0.9	4
F(ab)-9	F(ab)-8	<u>Gly</u> H49 <u>Ala</u>	CDR-H2 conformation	>150		2
F(ab)-10	F(ab)-8	AsnH76 <u>Ser</u>	Framework	6.4	1.2	4
F(ab)-11	F(ab)-10	LysH75 <u>Ala</u>	Framework	3.3	0.4	2
15 F(ab)-12	F(ab)-10	ArgH94 <u>Lys</u>	CDR-H3 conformation	1.6	0.6	4

<sup>a</sup>Anti-VEGF F(ab) variants were incubated with biotinylated VEGF and then transferred to ELISA plates coated with KDR-IgG (Park *et al.*, *supra*).

<sup>b</sup>Murine residues are underlined; residue numbers are according to Kabat *et al.*, *supra*.

20 <sup>c</sup>Mean and standard deviation are the average of the ratios calculated for each of the independent assays; the EC50 for chimeric F(ab) was 0.049 ± 0.013 mg/ml (1.0 nM).

Changing human residues H71 and H73 to their murine counterparts in F(ab)-4  
 25 improved binding by 4-fold (Table 2). Inspection of the models of the murine and humanized F(ab)s suggested that residue L46, buried at the VL-VH interface and interacting with CDR-H3 (Fig. 2), might also play a role either in determining the conformation of CDR-H3 and/or

affecting the relationship of the VL and VH domains. When the murine Val was exchanged for the human Leu at L46 (F(ab)-5), the binding affinity increased by almost 4-fold (Table 2). Three other buried framework residues were evaluated based on the molecular models: H49, H69 and H78. Position H69 may affect the conformation of CDR-H2 while position  
 5 H78 may affect the conformation of CDR-H1 (Figure 2). When each was individually changed from the human to murine counterpart, the binding improved by 2-fold in each case (F(ab)-6 and F(ab)-7, Table 2). When both were simultaneously changed, the improvement in binding was 8-fold (F(ab)-8, Table 2). Residue H49 was originally included as the murine Gly; when changed to the human consensus counterpart Ala the binding was reduced by 15-  
 10 fold (F(ab)-9, Table 2).

In F(ab)-10 and F(ab)-11 two residues in framework loop 3, FR-3, were changed to their murine counterparts: AsnH76 to murine Ser (F(ab)-10) and LysH75 to murine Ala (F(ab)-11). Both effected a relatively small improvement in binding (Table 2). Finally, at position H94 human and murine sequences most often have an Arg (Kabat *et al.*, *supra*). In  
 15 F(ab)-12, this Arg was replaced by the rare Lys found in the murine antibody (Fig. 1A) and this resulted in binding which was less than 2-fold from the chimeric F(ab) (Table 2). F(ab)-12 was also compared to the chimeric F(ab) using the BIAcore™ system (Pharmacia). Using this technique the  $K_d$  of the humanized F(ab)-12 was 2-fold weaker than that of the chimeric F(ab) due to both a slower  $k_{on}$  and faster  $k_{off}$  (Table 3).

20 **Table 3: Binding of Anti-VEGF F(ab) Variants to VEGF Using the BIAcore™ System<sup>a</sup>**

Variant	Amount of (Fab) bound (RU)	$k_{off}$ ( $s^{-1}$ )	$k_{on}$ ( $M^{-1}s^{-1}$ )	$K_d$ (nM)
chim-F(ab) <sup>b</sup>	4250	$5.9 \times 10^{-5}$	$6.5 \times 10^4$	0.91
F(ab)-12	3740	$6.3 \times 10^{-5}$	$3.5 \times 10^4$	1.8

25 <sup>a</sup> The amount of F(ab) bound, in resonance units (RU), was measured using a BIAcore™ system when 2  $\mu$ g F(ab) was injected onto a chip containing 2480 RU immobilized VEGF. Off-rate kinetics ( $k_{off}$ ) were measured by saturating the chip with F(ab) and then monitoring dissociation after switching to buffer. On-rate kinetics ( $k_{on}$ ) were measured using two-fold serial dilutions of F(ab).  $K_d$ , the equilibrium dissociation constant, was calculated as  $k_{off}/k_{on}$ .  
 30 <sup>b</sup> chim-F(ab) is a chimeric F(ab) with murine VL and VH domains fused to human CL and CH1 heavy domains.

Full length mAbs were constructed by fusing the VL and VH domains of the chimeric F(ab) and variant F(ab)-12 to the constant domains of human k light chain and human IgG1 heavy chain. The full length 12-IgG1 (F(ab)-12 fused to human IgG1) exhibited binding which was 1.7-fold weaker than the chimeric IgG1 (Table 4). Both 12-IgG1 and the chimeric IgG1 bound slightly less well than the original murine mAb A4.6.1 (Table 4).

**Table 4: Binding of Anti-VEGF IgG Variants to VEGF<sup>a</sup>**

IgG1/chIgG1 <sup>b</sup>			
Variant	Mean	S.D.	N
chIgG1	1.0		2
murIgG1 <sup>c</sup>	0.759	0.001	2
12-IgG1 <sup>d</sup>	1.71	0.03	2

<sup>a</sup>Anti-VEGF IgG variants were incubated with biotinylated VEGF and then transferred to ELISA plates coated with KDR-IgG (Park *et al.*, (1994), *supra*).

<sup>b</sup>chIgG1 is chimeric IgG1 with murine VL and VH domains fused to human CL and IgG1 heavy chains; the EC50 for chIgG1 was  $0.113 \pm 0.013$   $\mu\text{g/ml}$  (0.75 nM).

<sup>c</sup>murIgG1 is muMAbVEGF A461 purified from ascites.

<sup>d</sup>12-IgG1 is F(ab)-12 VL and VH domains fused to human CL and IgG1 heavy chains.

**Biological Studies:** rhuMAb VEGF and muMAb VEGF A.4.6.1. were compared for their ability to inhibit bovine capillary endothelial cell proliferation in response to a near maximally effective concentration of VEGF (3 ng/ml). As illustrated in Figure 3, the two MAbs were essentially equivalent, both in potency and efficacy. The ED50 values were respectively  $50 \pm 5$  ng/ml and  $48 \pm 8$  ng/ml ( $\sim 0.3$  nM). In both cases 90% inhibition was achieved at the concentration of 500 ng/ml ( $\sim 3$  nM). Neither muMAb VEGF A.4.6.1 nor rhuMAb VEGF had any effect on basal or bFGF-stimulated proliferation of capillary endothelial cells, confirming that the inhibition is specific for VEGF.

To determine whether such equivalency applies also to an *in vivo* system, the two antibodies were compared for their ability to suppress the growth of human A673 rhabdomyosarcoma cells in nude mice. Previous studies have shown that muMAb VEGF A.4.6.1 has a dramatic inhibitory effect in this tumor model (Kim *et al. Nature* 362:841-844

(1993) and Borgström *et al. Cancer Res* 56:4032-4039 (1996)). As shown in Figure 4, at both doses tested (0.5 and 5 mg/kg), the two antibodies markedly suppressed tumor growth as assessed by tumor weight measurements four weeks after cell inoculation. The decreases in tumor weight compared to the control group were respectively 85% and 93% at each dose  
5 in the animals treated with muMAb VEGF A.4.6.1. versus 90% and 95% in those treated with rhuMAb VEGF. Similar results were obtained with the breast carcinoma cell line MDA-MB 435.

## EXAMPLE 2

In this example, the murine anti-VEGF antibody A4.6.1 discussed above was  
10 humanized by randomizing a small set of framework residues and by monovalent display of the resultant library of antibody molecules on the surface of filamentous phage in order to identify high affinity framework sequences via affinity-based selection.

### MATERIALS AND METHODS

**Construction of Anti-VEGF Phagemid Vector, pMB4-19:** The murine anti-VEGF  
15 mAb A4.6.1 is discussed above in Example 1. The first Fab variant of humanized A4.6.1, hu2.0, was constructed by site-directed mutagenesis using a deoxyuridine-containing template of plasmid pAK2 (Carter *et al. Proc. Natl. Acad. Sci. U.S.A.* 89:4285-4289 (1992)) which codes for a human V<sub>L</sub>κI-Cκ<sub>1</sub> light chain and human V<sub>H</sub>III-C<sub>H</sub>Iγ<sub>1</sub> heavy chain Fd fragment. The transplanted A4.6.1 CDR sequences were chosen according to the sequence  
20 definition of Kabat *et al., supra*, except for CDR-H1 which included residues 26-35. The Fab encoding sequence was subcloned into the phagemid vector phGHamg3 (Bass *et al. Proteins* 8:309-314 (1990) and Lowman *et al. Biochemistry* 30:10832-10838 (1991)). This construct, pMB4-19, encodes the initial humanized A4.6.1 Fab, hu2.0, with the C-terminus of the heavy chain fused precisely to the carboxyl portion of the M13 gene III coat protein. pMB4-19 is  
25 similar in construction to pDH188, a previously described plasmid for monovalent display of Fab fragments (Garrard *et al. Biotechnology* 9:1373-1377 (1991)). Notable differences between pMB4-19 and pDH188 include a shorter M13 gene III segment (codons 249-406) and use of an amber stop codon immediately following the antibody heavy chain Fd fragment. This permits expression of both secreted heavy chain or heavy chain-gene III fusions in *supE*  
30 suppressor strains of *E. coli*.

**Expression and Purification of Humanized A4.6.1 Fab Fragment:** *E. coli* strain 34B8, a nonsuppressor, was transformed with phagemid pMB4-19, or variants thereof.

Single colonies were grown overnight at 37°C in 5 mL 2YT containing 50 µg/mL carbenicillin. These cultures were diluted into 200 mL AP5 medium (Chang *et al. Gene* 55:189-196 (1987)) containing 20 µg/mL carbenicillin and incubated for 26 hr at 30°C. The cells were pelleted at 4000 x g and frozen at -20°C for at least 2 h. Cell pellets were then  
 5 resuspended in 5 mL of 10 mM Tris-HCl (pH 7.6) containing 1 mM EDTA, shaken at 4°C for 90 min and centrifuged at 10,000 x g for 15 min. The supernatant was applied to a 1 mL streptococcal protein G-sepharose column (Pharmacia) and washed with 10 mL of 10 mM MES (pH 5.5). The bound Fab fragment was eluted with 2.5 mL 100 mM acetic acid and immediately neutralized with 0.75 mL 1M Tris-HCl, pH 8.0. Fab preparations were buffer-  
 10 exchanged into PBS and concentrated using Centricon-30 concentrators (Amicon). Typical yields of Fab were ~1 mg/L culture, post-protein G purification. Purified Fab samples were characterized by electrospray mass spectrometry, and concentrations were determined by amino acid analysis.

**Construction of the Anti-VEGF Fab Phagemid Library:** The humanized A4.6.1  
 15 phagemid library was constructed by site-directed mutagenesis according to the method of Kunkel *et al. Methods Enzymol.* 204:125-139 (1991)). A derivative of pMB4-19 containing TAA stop triplets at V<sub>H</sub> codons 24, 37, 67 and 93 was prepared for use as the mutagenesis template (all sequence numbering according to Kabat *et al., supra*). This modification was to prevent subsequent background contamination by wild type sequences. The codons  
 20 targeted for randomization were 4 and 71 (light chain) and 24, 37, 67, 69, 71, 73, 75, 76, 78, 93 and 94 (heavy chain).

In order to randomize heavy chain codons 67, 69, 71, 73, 75, 76, 78, 93 and 94 with a single mutagenic oligonucleotide, two 126-mer oligonucleotides were first preassembled from 60 and 66-mer fragments by template-assisted enzymatic ligation. Specifically, 1.5 nmol  
 25 of 5' phosphorylated oligonucleotide 503-1 (5'-GAT TTC AAA CGT CGT NYT ACT WTT TCT AGA GAC AAC TCC AAA AAC ACA BYT TAC CTG CAG ATG AAC-3' (SEQ ID NO:22)) or 503-2 (5'-GAT TTC AAA CGT CGT NYT ACT WTT TCT TTA GAC ACC TCC GCA AGC ACA BYT TAC CTG CAG ATG AAC-3' (SEQ ID NO:23)) were combined with 1.5 nmol of 503-3 (5'-AGC CTG CGC GCT GAG GAC ACT GCC GTC  
 30 TAT TAC TGT DYA ARG TAC CCC CAC TAT TAT GGG-3' (SEQ ID NO:24)) (randomized codons underlined; N=A/G/T/C; W=A/T; B=G/T/C; D=G/A/T; R=A/G; Y=C/T). Then, 1.5 nmol of template oligonucleotide (5'-CTC AGC GCG CAG GCT GTT



CAT CTG CAG GTA-3' (SEQ ID NO:25)), with complementary sequence to the 5' ends of 503-1/2 and the 3' end of 503-3, was added to hybridize to each end of the ligation junction. *Taq* ligase (thermostable ligase from New England Biolabs) and buffer were added, and the reaction mixture was subjected to 40 rounds of thermal cycling, (95°C 1.25 min; 50°C for 5 min) so as to cycle the template oligonucleotide between ligated and unligated junctions. The product 126-mer oligonucleotides were purified on a 6% urea/TBE polyacrylamide gel and extracted from the polyacrylamide in buffer. The two 126-mer products were combined in equal ratio, ethanol precipitated and finally solubilized in 10mM Tris-HCl, 1mM EDTA. The mixed 126-mer oligonucleotide product was labeled 504-01.

Randomization of select framework codons ( $V_L$  4, 71;  $V_H$  24, 37, 67, 69, 71, 73, 75, 76, 93, 94) was effected in two steps. Firstly,  $V_L$  randomization was achieved by preparing three additional derivatives of the modified pMB4-19 template. Framework codons 4 and 71 in the light chain were replaced individually or pairwise using the two mutagenic oligonucleotides 5'-GCT GAT ATC CAG TTG ACC CAG TCC CCG-3' (SEQ ID NO:26) and 5'-and TCT GGG ACG GAT TAC ACT CTG ACC ATC-3' (SEQ ID NO:27). Deoxyuridine-containing template was prepared from each of these new derivatives. Together with the original template, these four constructs coded for each of the four possible light chain framework sequence combinations (Table 5).

Oligonucleotides 504-1, a mixture of two 126-mer oligonucleotides (see above), and 5'-CGT TTG TCC TGT GCA RYT TCT GGC TAT ACC TTC ACC AAC TAT GGT ATG AAC TGG RTC CGT CAG GCC CCG GGT AAG-3' (SEQ ID NO:28) were used to randomize heavy chain framework codons using each of the four templates just described. The four libraries were electroporated into *E. coli* XL-1 Blue cells (Stratagene) and combined. The total number of independent transformants was estimated at  $>1.2 \times 10^8$ , approximately 1,500-fold greater than the maximum number of DNA sequences in the library.

A variety of systems have been developed for the functional display of antibody fragments on the surface of filamentous phage. Winter *et al.*, *Ann. Rev. Immunol.* 12,433 (1994). These include the display of Fab or single chain Fv (scFv) fragments as fusions to either the gene III or gene VIII coat proteins of M13 bacteriophage. The system selected herein is similar to that described by Garrard *et al.*, *Biotechnol.* 9,1373 (1991) in which a Fab fragment is monovalently displayed as a gene III fusion (Figure 7). This system has two notable features. In particular, unlike scFvs, Fab fragments have no tendency to form dimeric

species, the presence of which can prevent selection of the tightest binders due to avidity effects. Additionally the monovalency of the displayed protein eliminates a second potential source of avidity effects that would otherwise result from the presence of multiple copies of a protein on each phagemid particle. Bass and Wells, *Proteins* 8:309 (1990) and Lowman  
5 *et al.*, *Biochemistry* 30:10832 (1991).

Phagemid particles displaying the humanized A4.6.1 Fab fragments were propagated in *E. coli* XL-1 Blue cells. Briefly, cells harboring the randomized pMB4-19 construct were grown overnight at 37°C in 25 mL 2YT medium containing 50 µg/mL carbenicillin and approximately 10<sup>10</sup> M13KO7 helper phage (Vieira & Messing *Methods Enzymol.* 153:3-11  
10 (1987)). Phagemid stocks were purified from culture supernatants by precipitation with a saline polyethylene glycol solution, and resuspended in 100 µL PBS (~10<sup>14</sup> phagemid/mL)

**Selection of Humanized A4.6.1 Fab Variants:** Purified VEGF<sub>121</sub> (100 µL at 10 µg/mL in PBS) was coated onto a microtiter plate well overnight at 4°C. The coating solution was discarded and this well, in addition to an uncoated well, were blocked with 6% skim milk for 1 h and washed with PBS containing 0.05% TWEEN 20™ (detergent). Then,  
15 10 µL of phagemid stock, diluted to 100 µL with 20 mM Tris (pH 7.5) containing 0.1% BSA and 0.05% TWEEN 20™, was added to each well. After 2 hours the wells were washed and the bound phage eluted with 100 µL of 0.1 M glycine (pH 2.0), and neutralized with 25 µL of 1M Tris pH 8.0. An aliquot of this was used to titer the number of phage eluted. The  
20 remaining phage eluted from the VEGF-coated well were propagated for use in the next selection cycle. A total of 8 rounds of selection was performed after which time 20 individual clones were selected and sequenced (Sanger *et al. Proc. Natl. Acad. Sci. U.S.A.* 74:5463-5467 (1977)).

**Determination of VEGF Binding Affinities:** Association (k<sub>on</sub>) and dissociation (k<sub>off</sub>)  
25 rate constants for binding of humanized A4.6.1 Fab variants to VEGF<sub>121</sub> were measured by surface plasmon resonance (Karlsson *et al. J. Immun. Methods* 145:229-240 (1991)) on a Pharmacia BIAcore instrument. VEGF<sub>121</sub> was covalently immobilized on the biosensor chip via primary amino groups. Binding of humanized A4.6.1 Fab variants was measured by flowing solutions of Fab in PBS/0.05% TWEEN 20™ over the chip at a flow rate of 20  
30 µL/min. Following each binding measurement, residual Fab was stripped from the immobilized ligand by washing with 5 µL of 50 mM aqueous HCl at 3 µL/min. Binding

profiles were analyzed by nonlinear regression using a simple monovalent binding model (BIAevaluation software v2.0; Pharmacia).

### RESULTS

5        **Construction of Humanized A4.6.1:** An initial humanized A4.6.1 Fab fragment was constructed (hu2.0, Figs. 5A and 5B), in which the CDRs from A4.6.1 were grafted onto a human  $V_L\kappa I$ - $V_H III$  framework. All other residues in hu2.0 were maintained as the human sequence. Binding of this variant to VEGF was so weak as to be undetectable. Based on the relative affinity of other weakly-binding humanized A4.6.1 variants, the  $K_D$  for binding of  
10 hu2.0 was estimated at  $>7 \mu M$ . This contrasts with an affinity of 1.6 nM for a chimeric Fab construct consisting of the intact  $V_L$  and  $V_H$  domains from murine A4.6.1 and human constant domains. Thus binding of hu2.0 to VEGF was at least 4000-fold reduced relative to the chimera.

**Design of Antibody Library:** The group of framework changes to the human  
15 framework sequence herein is shown in Table 5 and Fig. 6.

**Table 5: Key Framework Residues Important for Antigen Binding and Targeted for Randomization**

20	Framework residue		Human $V_{L\kappa I}$ , $V_{H III}$ consensus residue	Murine A4.6.1 residue	Randomization <sup>a</sup>
	$V_L$ :	4	Met	Met	Met, Leu
		71	Phe	Tyr	Phe, Tyr
	$V_H$ :	24	Ala	Ala	Ala, Val, Thr
		37	Val	Val	Val, Ile
25		67	Phe	Phe	Phe, Val, Thr, Leu, Ile, Ala
		69	Ile	Phe	Ile, Phe
		71	Arg	Leu	Arg <sup>b</sup> , Leu <sup>b</sup>
		73	Asp	Thr	Asp <sup>b</sup> , Thr <sup>b</sup>
		75	Lys	Ala	Lys <sup>b</sup> , Ala <sup>b</sup>
30		76	Asn	Ser	Asn <sup>b</sup> , Ser <sup>b</sup>

20	Framework residue		Human V $\kappa$ <sub>L</sub> I, V <sub>H</sub> III consensus residue	Murine A4.6.1 residue	Randomization <sup>a</sup>
		78	Leu	Ala	Leu, Ala, Val, Phe
		93	Ala	Ala	Ala, Val, Leu, Ser, Thr
		94	Arg	Lys	Arg, Lys

<sup>a</sup>Amino acid diversity in phagemid library

- 5 <sup>b</sup>V<sub>H</sub>71, 73, 75, 76 randomized to yield the all-murine (L71/T73/A75/S76) or all-human (R71/D73/K75/N76) V<sub>H</sub>III tetrad

A concern in designing the humanized A4.6.1 phagemid library was that residues targeted for randomization were widely distributed across the V<sub>L</sub> and V<sub>H</sub> sequences.

10 Limitations in the length of synthetic oligonucleotides requires that simultaneous randomization of each of these framework positions can only be achieved through the use of multiple oligonucleotides. However, as the total number of oligonucleotides increases, the efficiency of mutagenesis decreases (*i.e.* the proportion of mutants obtained which incorporate sequence derived from all of the mutagenic oligonucleotides). To circumvent

15 this problem, two features were incorporated into the library construction. The first was to prepare four different mutagenesis templates coding for each of the possible V<sub>L</sub> framework combinations. This was simple to do given the limited diversity of the light chain framework (only 4 different sequences), but was beneficial in that it eliminated the need for two oligonucleotides from the mutagenesis strategy. Secondly, two 126-base oligonucleotides

20 were preassembled from smaller synthetic fragments. This made possible randomization of V<sub>H</sub> codons 67, 69, 71, 73, 75, 76, 93 and 94 with a single long oligonucleotide, rather than two smaller ones. The final randomization mutagenesis strategy therefore employed only two oligonucleotides simultaneously onto four different templates.

*Selection of Tight Binding Humanized A4.6.1 Fab's:* Variants from the humanized

25 A4.6.1 Fab phagemid library were selected based on binding to VEGF. Enrichment of functional phagemid, as measured by comparing titers for phage eluted from a VEGF-coated versus uncoated microtiter plate well, increased up to the seventh round of affinity panning. After one additional round of sorting, 20 clones were sequenced to identify preferred framework residues selected at each position randomized. These results,

30 summarized in Table 6, revealed strong consensus amongst the clones selected. Ten out of

the twenty clones had the identical DNA sequence, designated hu2.10. Of the thirteen framework positions randomized, eight substitutions were selected in hu2.10 ( $V_L$  71;  $V_H$  37, 71, 73, 75, 76, 78 and 94). Interestingly, residues  $V_H$  37 (Ile) and 78 (Val) were selected neither as the human  $V_H$ III or murine A4.6.1 sequence. This result suggests that

5 some framework positions may benefit from extending the diversity beyond the target human and parent murine framework sequences.

**Table 6: Sequences Selected from the Humanized A4.6.1 Phagemid Fab Library**

10	<b>Variant</b>	<b>Residue substitutions</b>												
		$V_L$		$V_H$										
		4	71	24	37	67	69	71	73	75	76	78	93	94
	murine A4.6.1	M	Y	A	V	F	F	L	T	A	S	A	A	K
15	hu2.0 (CDR-graft)	M	<u>E</u>	A	V	F	<u>I</u>	<u>R</u>	<u>N</u>	<u>K</u>	<u>N</u>	<u>L</u>	A	<u>R</u>
<b>Phage-selected clones:</b>														
	hu2.1(2)	-	Y	-	I	-	-	-	-	-	-	V	-	K
	hu2.2(2)	L	Y	-	I	-	-	-	-	-	-	V	-	K
20	hu2.6(1)	L	-	-	I	T	-	L	T	A	S	V	-	K
	hu2.7(1)	L	-	-	I	-	-	-	-	-	-	V	-	K
	hu2.10(10)	-	Y	-	I	-	-	L	T	A	S	V	-	K

Differences between hu2.0 and murine A4.6.1 antibodies are underlined. The number of identical clones identifies for each phage-selected sequence is indicated in parentheses.

25 Dashes in the sequences of phage-selected clones indicate selection of the human  $V_L$ KI- $V_H$ III framework sequence (*i.e.* as in hu2.0).

There were four other unique amino acid sequences among the remaining ten clones

30 analyzed: hu2.1, hu2.2, hu2.6 and hu2.7. All of these clones, in addition to hu2.10, contained identical framework substitutions at positions  $V_H$  37 (Ile), 78 (Val) and 94 (Lys), but retained the human  $V_H$ III consensus sequence at positions 24 and 93. Four clones had lost the light chain coding sequence and did not bind VEGF when tested in a phage ELISA

assay (Cunningham *et al.* *EMBO J.* 13:2508-251 (1994)). Such artifacts can often be minimized by reducing the number of sorting cycles or by propagating libraries on solid media.

**Expression and Binding Affinity of Humanized A4.6.1 Variants:** Phage-selected variants hu2.1, hu2.2, hu2.6, hu2.7 and hu2.10 were expressed in *E. coli* using shake flasks and Fab fragments were purified from periplasmic extracts by protein G affinity chromatography. Recovered yields of Fab for these five clones ranged from 0.2 (hu2.6) to 1.7 mg/L (hu2.1). The affinity of each of these variants for antigen (VEGF) was measured by surface plasmon resonance on a BIAcore instrument (Table 7). Analysis of this binding data revealed that the consensus clone hu2.10 possessed the highest affinity for VEGF out of the five variants tested. Thus the Fab phagemid library was selectively enriched for the tightest binding clone. The calculated  $K_D$  for hu2.10 was 55 nM, at least 125-fold tighter than for hu2.0 which contains no framework changes ( $K_D > 7 \mu\text{M}$ ). The other four selected variants all exhibited weaker binding to VEGF, ranging down to a  $K_D$  of 360 nM for the weakest (hu2.7). Interestingly, the  $K_D$  for hu2.6, 67 nM, was only marginally weaker than that of hu2.10 and yet only one copy of this clone was found among 20 clones sequenced. This may have due to a lower level of expression and display, as was the case when expressing the soluble Fab of this variant. However, despite the lower expression rate, this variant is useful as a humanized antibody.

**Table 7: VEGF Binding Affinity of Humanized A4.6.1 Fab Variants**

Variant	$k_{on}$ $\text{M}^{-1}\text{s}^{-1}/10^4$	$k_{off}$ $10^4\text{s}^{-1}$	$K_D$ nM	$\frac{K_D(\text{A4.6.1})}{K_D(\text{mut})}$
A4.6.1 chimera	5.4	0.85	1.6	>4000
hu2.0	ND	ND	>7000**	
<b>Phage selected clones:</b>				
hu2.1	0.70	18	260	170
hu2.2	0.47	16	340	210
hu2.6	0.67	4.5	67	40
hu2.7	0.67	24	360	230
hu2.10	0.63	3.5	55	35
*hu2.10V	2.0	1.8	9.3	5.8

\*hu2.10V = hu2.10 with mutation V<sub>L</sub> Leu→Val

Estimated errors in the Biacore binding measurements are +/-25%.

\*\*Too weak to measure; estimate of lower bound

- 5        *Additional Improvement of Humanized Variant hu2.1:* Despite the large improvement in antigen affinity over the initial humanized variant, binding of hu2.10 to VEGF was still 35-fold weaker than a chimeric Fab fragment containing the murine A4.6.1 V<sub>L</sub> and V<sub>H</sub> domains. This considerable difference suggested that further optimization of the humanized framework might be possible through additional mutations. Of the Vernier
- 10 residues identified by Foote & Winter *J. Mol. Biol.* 224:487-499 (1992), only residues V<sub>L</sub> 46, V<sub>H</sub> 2 and V<sub>H</sub> 48 differed in the A4.6.1 versus human V<sub>L</sub>κI-V<sub>H</sub>III framework (Figs. 5A and 5B) but were not randomized in our phagemid library. A molecular model of the humanized A4.6.1 Fv fragment showed that V<sub>L</sub> 46 sits at the V<sub>L</sub>-V<sub>H</sub> interface and could influence the conformation of CDR-H3. Furthermore, this amino acid is almost always
- 15 leucine in most V<sub>L</sub>κ frameworks (Kabat *et al.*, *supra*), but is valine in A4.6.1. Accordingly, a Leu → Val substitution was made at this position in the background of hu2.10. Analysis of binding kinetics for this new variant, hu2.10V, indicated a further 6-fold improvement in the K<sub>D</sub> for VEGF binding, demonstrating the importance of valine at position V<sub>L</sub> 46 in antibody A4.6.1. The K<sub>D</sub> for hu2.10V (9.3 nM) was thus within 6-fold that of the chimera.
- 20 In contrast to V<sub>L</sub> 46, no improvement in the binding affinity of hu2.10 was observed for replacement of either V<sub>H</sub> 2 or V<sub>H</sub> 48 with the corresponding residue from murine A4.6.1.

### EXAMPLE 3

- In this example, CDR randomization, affinity maturation by monovalent Fab phage display, and cumulative combination of mutations were used to enhance the affinity of a
- 25 humanized anti-VEGF antibody.

- Construction of Humanized Antibody pY0101:* Phage-displayed antibody vector phMB4-19-1.6 (see Figs. 8A-E) was used as a parent. In this construct, anti-VEGF is expressed as a Fab fragment with its heavy chain fused to the N-terminus of the truncated
- 30 g3p. Both the light and heavy chains are under the control of phoA promoter with an upstream stII signal-sequence for secretion into the periplasm. Point mutations outside the CDR regions were made by site-directed mutagenesis to improve affinity for VEGF with

oligonucleotides HL-242, HL-243, HL-245, HL-246, HL-254, HL-256, and HL-257 as shown in Table 8 below:

*Table 8: Oligos for Directed Mutations*

5	<b>Oligo Number</b>	<b>Region</b>	<b>Substitution/ Comments</b>	<b>Sequence</b>
	HL-242	VL	M4L	5'-GATATCCAGTTGACCCAGTCCCCG-3' (SEQ ID NO:29)
	HL-243	VL	L46V	5'-GCTCCGAAAGTACTGATTTAC-3' (SEQ ID NO:30)
	HL-245	VH	CDR-7	5'-CGTCGTTTCACTTTTTCTGCAGACACCT CCAGCAACACAGTATACCTGCAGATG-3' (SEQ ID NO:31)
10	HL-246	VH	R98K	5'-CTATTACTGTGCAAAGTACCCCCAC-3' (SEQ ID NO:32)
	HL-254	VL	Y71F	5'-GGGACGGATTTCACTCTGACCATC-3' (SEQ ID NO:33)
	HL-256	VH	I37V	5'-GGTATGAACTGGGTCCGTCAGGCCCC-3' (SEQ ID NO:34)
	HL-257	VH	CDR-7 A72L S76K N77S	5'-CGTCGTTTCACTTTTTCTTTAGACACCT CCAAAGACAGCATACCTGCAGATGAA C-3' (SEQ ID NO:35)

15 The resulting variant was termed Y0101 (Figs. 9A and 9B).

*Construction of the First Generation of Antibody-Phage Libraries:* To prevent contamination by wild-type sequence, templates with the TAA stop codon at the targeted sites for randomization were prepared and used for constructing libraries by site-directed mutagenesis with oligonucleotides using the degenerate NNS codon (where N is an equal mixture of A, G, C, and T while S is an equal mixture of G and C) for saturation mutagenesis. VL1 and VH3 were chosen as potential candidates for affinity enhancement (Figs. 9A and B). Within the CDRs, two libraries were constructed from the pY0101 template. VL1 was mutated using stop-template oligonucleotides HL-248 and HL-249 (Table 9) and library oligonucleotides HL-258 and HL-259 (Table 10). Similarly, three libraries were constructed for VH3 using stop template oligonucleotides HL-250, HL-251, and HL-252 (Table 9), and library oligonucleotides HL-260, HL-261, and HL-262 (Table 10). Library construction is summarized in Tables 9 and 10 below.



*Table 9: Template Oligos for Mutagenesis*

Oligo Number	Region Comments	Sequence
HL-248	VL1	5'-GGGTCACCATCACCTGCTAAGCATAATAATAA TAAAGCAACTATTTAAACTGG-3' (SEQ ID NO:36)
HL-249	VL1	5'-GCGCAAGTCAGGATATTTAATAATAATAATAA TGGTATCAACAGAAACCAGG-3' (SEQ ID NO:37)
HL-250	VH3	5'-GTCTATTACTGTGCAAAGTAATAACACTAATA AGGGAGCAGCCACTGG-3' (SEQ ID NO:38)
HL-251	VH3	5'-GGTACCCCCACTATTATTAATAATAATAATGG TATTTCGACGTCTGGGG-3' (SEQ ID NO:39)
HL-252	VH3	5'-CACTATTATGGGAGCAGCCACTAATAATAATA AGTCTGGGTCAAGGAACCCTG-3' (SEQ ID NO:40)
HL-263	VH1	5'-TCCTGTGCAGCTTCTGGCTAATAATTCTAATA ATAAGGTATGAACTGGGTCCG-3' (SEQ ID NO:41)
HL-264	VH2	5'-GAATGGGTTGGATGGATTAATAATAATAAG GTTAACCGACCTATGCTGCGG-3' (SEQ ID NO:42)
YC-80	VH3	5'-CTGTGCAAAGTACCCGTAATATTAATAATAAT AACACTGGTATTTTCGAC-3' (SEQ ID NO:43)
YC-100	CDR7	5'-CGTTTCACTTTTTCTTAAGACTAATCCAAATA AACAGCATACCTGCAG-3' (SEQ ID NO:44)
YC-102	VH2	5'-GAATGGGTTGGATGGATTTAATAATAATAAG GTGAACCGACCTATG-3' (SEQ ID NO:45)

Table 10: Random Oligos for Library Construction

Oligo Number	Region Comment	Sequence
5 HL-258	VL1	5'-GGGTCACCATCACCTGCNNSGCANNNSNNSNNSN SAGC AACTATTTAAACTGG-3' (SEQ ID NO:46)
HL-259	VL1	5'-GCGCAAGTCAGGATATTNNSNNSNNSNNSNNSNSTG GTATCAACAGAAACCAGG-3' (SEQ ID NO:47)
HL-260	VH3	5'-GTCTATTACTGTGCAAAGNNSNNSCACNNSNNSG GGAGCAGCCACTGG-3' (SEQ ID NO:48)
HL-261	VH3	5'-TACCCCCACTATTATNNSNNSNNSNNSNSTGGTATTT CGACGTCTGGGG-3' (SEQ ID NO:49)
HL-262	VH3	5'-CACTATTATGGGAGCAGCCACNNSNNSNNSNNSG TCTGGGGTCAAGGAACCCTG-3' (SEQ ID NO:50)
HL-265	VH1	5'-TCCTGTGCAGCTTCTGGCNNSNNSSTTCNNSNNSN NSGGTATGAACTGGGTCCG-3' (SEQ ID NO:51)
10 HL-266	VH2	5'-GAATGGGTTGGATGGATTAAACNNSNNSNNSGGTN NSCCGACCTATGCTGCGG-3' (SEQ ID NO:52)
YC-81	VH3	5'-CTGTGCAAAGTACCCGNNSTATNNSNNSNNSNNS CACTGGTATTTTCGAC-3' (SEQ ID NO:53)
YC-101	CDR7	5'-CGTTTCACTTTTTCTNNSGACNNSTCCAAANNSA CAGCATACCTGCAG-3' (SEQ ID NO:54)
YC-103	VH2	5'-GAATGGGTTGGATGGATTNNSNNSNNSNNSGGTG AACCGACCTATG-3' (SEQ ID NO:55)

15

The products of random mutagenesis reactions were electroporated into XL1-Blue *E.coli* cells (Stratagene) and amplified by growing 15-16 h with M13KO7 helper phage. The complexity of each library, ranging from  $2 \times 10^7$  to  $1.5 \times 10^8$ , was estimated based upon plating of the initial transformation onto carbenicillin plates.

20

**Initial Affinity Selections:** For each round of selection, approximately  $10^9$ - $10^{10}$  phage were screened for binding to plates (Nunc Maxisorp 96-well) coated with 2  $\mu$ g/mL VEGF (recombinant; residue 9-109 version) in 50 mM carbonate buffer, pH 9.6 and blocked with 5% instant milk in 50 mM carbonate buffer, pH 9.6. After 1-2 hour binding at room temperature, in the presence of 0.5% bovine serum albumin and 0.05% TWEEN 20™ in

25 PBS, the phage solution was removed, and the plate was washed ten times with PBS/TWEEN™ (0.05% TWEEN 20™ in PBS buffer). Typically, to select for enhanced

affinity variants with slower dissociation rates, the plates were incubated with PBS/TWEEN™ buffer for a period of time which lengthened progressively for each round of selection (from 0 minute for the first round, to 3 h for the ninth round of selection). After the PBS/TWEEN™ buffer was removed, the remained phages were eluted with 0.1 M HCl  
5 and immediately neutralized with 1/3 volume of 1 M Tris, pH 8.0. The eluted phages were propagated by infecting XL1-Blue *E.coli* cells (Stratagene) for the next selection cycle.

Sequencing data revealed that both VL1 libraries, even after the eighth/ninth round of sorting, remained diverse, tolerating various type of residues at the sites of randomization. In contrast, the VH3 libraries retained only wild type residues or had very conservative  
10 substitutions. This suggested that the VL1 was more exposed to solvent and lay outside the binding interface. In contrast, VH3 did not show dramatically different sidechain substitutions, and therefore might be more intimately involved in antigen binding.

*Phage-ELISA Assay of Binding Affinities:* From each of these libraries, representative clones (those represented by abundant sequences) were assayed for their  
15 affinities relative to that of parent clone pY0101 in a phage-ELISA assay. In such an assay, phages were first serially diluted to determine a fractional saturation titer which was then held constant and used to incubate with varying concentrations of VEGF (starting at 200 nM to 0 nM) in solution. The mixture was then transferred onto plate precoated with VEGF (2 µg/mL) and blocked with 5% instant milk, and allowed to equilibrate for 1 hour at room  
20 temperature. Thereafter, the phage solution was removed and the remaining bound phages were detected with a solution of rabbit anti-phage antibody mixed with goat anti-rabbit conjugate of horse radish peroxidase. After an hour incubation at room temperature, the plate was developed with a chromogenic substrate, *o*-phenylenediamine (Sigma). The reaction was stopped with addition of 1/2 volume of 2.5 M H<sub>2</sub>SO<sub>4</sub>. Optical density at 492nm  
25 was measured on a spectrophotometric plate reader.

Although all of the selected clones from these five libraries showed either weaker or similar affinities than that of wild type pY0101 in phage-ELISA assay, one particular variant (pY0192) from library HL-258 displayed an apparent advantage (about 10 fold) in the level of expression or phage display relative to pY0101. This clone contained mutations S24R,  
30 S26N, Q27E, D28Q, and I29L in the VL region (Fig. 9A). In addition, this variant was found to have a spurious mutation, M34I, in VH. This variant showed no significant difference in binding affinity to VEGF as compared with the pY0101 variant. To improve

the level of Fab-display on phage, and the signal-to-noise ratio for phage-ELISA assays, the corresponding substitutions in pY0192 at VL1 were incorporated into the template background for constructing both CDR Ala-mutants and the second generation of anti-VEGF libraries.

- 5        *Ala-Scanning the CDRs of Anti-VEGF:* To determine the energetics contributed by each of the amino acids in the CDR regions and thus better select target residues for randomization, the CDR regions were screened by substituting alanine for each residue. Each Ala mutant was constructed using site-directed mutagenesis with a synthetic oligonucleotide encoding for the specific alanine substitution. Where Ala was the wild-type residue, Ser was substituted to test the effect of a sidechain substitution. Phage clones having  
10       a single Ala mutation were purified and assayed in phage-ELISA as described above. Results of the Ala-scan demonstrated that Ala-substitution at various positions can have an effect, ranging from 2 to > 150 fold reductions, on antigen binding affinity compared to pY0192. In addition, it confirmed a previous observation that VH3, but not VL1, was involved in  
15       antigen binding. Results of the CDR Ala-scan are summarized in Table 11 below.

*Table 11: Relative VEGF Affinities of Ala-Scan Fab Variants*

	Residue VL	IC50 (mut) IC50 (wt)	Residue VH	IC50 (mut) IC50 (wt)
20	R24A	1	G26A	2
	A25S	1	Y27A	34
	N26A	1	T28A	1
	E27A	1	F29A	16
	Q28A	1	T30A	1
25	L29A	1	N31A	>150
	S30A	2	Y32A	>150
	N31A	2	G33A	6
	Y32A	2	I34A	6
	L33A	2	N35A	66
30	N34A	4		
			W50A	>150
	F50A	1	I51A	4
	T51A	1	N52A	>150
	S52A	1	T53A	9
35	S53A	1	Y54A	9
	L54A	1	T55A	4
	H55A	1	G56A	1

	Residue VL	IC50 (mut) IC50 (wt)	Residue VH	IC50 (mut) IC50 (wt)
	S56A	1	E57A	2
			P58A	1
	Q89A	4	T59A	3
	Q90A	3	Y60A	2
5	Y91A	14	A61S	1
	S92A	1	A62S	1
	T93A	1	D63A	1
	V94A	2	F64A	1
	P95A	3	K65A	1
10	W96A	>150	R66A	1
	T97A	1		
			Y99A	>150
			P100A	38
			H101A	4
15			Y102A	4
			Y103A	5
			G104A	2
			S105A	1
			S106A	>150
20			H107A	2
			W108A	>150
			Y109A	19
			F110A	25
			D111A	2

25

All variants are in the background of pY0192 ("wt"; see Figs. 9A-B). IC50's were determined in a competitive phage-ELISA assay.

The largest effects of Ala substitutions are seen in CDRs H1, H2, and H3, including  
 30 Y27A (34-fold reduction in affinity), N31A, Y32A, W50A, N52A, Y99A, S106A and  
 W108A (each >150-fold reduction); N35A (66-fold reduction), P100A (38-fold reduction)  
 and F110A (25-fold reduction). In contrast, only one VL substitution had a large impact on  
 binding affinity, W96A (>150-fold reduction). These results point to the three VH CDRs as  
 the main energetic determinants of Fab binding to VEGF, with some contribution from VL3.

35 ***Design of Second-Generation CDR Mutation Libraries:*** Two additional libraries  
 which randomized existing residues in anti-VEGF version Y0192 were designed based upon  
 inspection of the crystal structure. In VH2, residues 52-55 were randomized because they

lie within the binding interface with VEGF. An additional region of the Fab, termed "CDR7" (see Fig. 10B), was also targeted for randomization because several residues in this loop, while not contacting VEGF, do have contacts with the VH loops of the antibody. These represented potential sites for affinity improvement through secondary effects upon the interface residues. Residues L72, T74, and S77 were randomized in this CDR7 library.

Also based upon the crystal structure, one of the original CDR libraries was reconstructed to re-test the potential for affinity maturation in the VH1 CDR. Residues 27, 28, and 30-32 were randomized using the new Y0192 background.

**Second-Generation Selections of Anti-VEGF Libraries:** Based on Ala-scan results as well as the crystal structure of the antigen-antibody (F(ab)-12) complex, a total of seventeen libraries were constructed using the pY0192 template and stop-template oligonucleotides (which code for a stop codon at the sites targeted for randomization) YC-80, YC-100, YC-102, HL-263, and HL-264 (Table 9 above). The corresponding randomization oligonucleotides (which employ NNS at the sites targeted for randomization) were YC81, YC-101, YC-103, HL-265, and HL-266 (Table 10 above). The resulting transformants yielded libraries with complexities ranging from  $6 \times 10^7$  to  $5 \times 10^8$  which suggests that the libraries were comprehensive in covering all possible variants. Phage libraries were sorted for 7-8 rounds using conditions as described in Table 12 below.

**Table 12: Conditions for Secondary Selections of Fab Variants**

Round of Selection	Incubation Time (hr)	Incubation Solution	Incubation Temp. (°C)
1	0	0	room temp.
2	1	ELISA buffer	room temp.
3	2	1 $\mu$ M VEGF/ELISA	room temp.
4	18	1 $\mu$ M VEGF/ELISA	room temp.
5	37	1 $\mu$ M VEGF/ELISA	room temp.
6	17 hr @ room temp./ 30 hr @ 37°C	1 $\mu$ M VEGF/ELISA	room temp./37°C
7	63	1 $\mu$ M VEGF/ELISA	37°C
8	121	1 $\mu$ M VEGF/ELISA	37°C

ELISA buffer contained 0.5% bovine serum albumin and 0.05% TWEEN 20™ in PBS. VEGF was included in the incubation buffer to minimize rebinding of phages to VEGF coated on the surface of the plate. Sorting of these libraries yielded phage enrichments over 7 to 8 rounds of selection.

- 5 *Phage-ELISA Assays of Second Generation Clones:* After eight round of selections, ten to twenty clones from each library were isolated from carbenicillin containing plates harboring *E. coli* (XL1) colonies which had been infected with an eluted phage pool. Colonies were isolated and grown with helper phage to obtain single-stranded DNA for sequencing. CDR substitutions selected for more favorable binding to VEGF were deduced  
10 from the DNA sequences of phagemid clones. A sampling of selected clones is shown in Table 13 below.

*Table 13: Protein Sequences of Anti-VEGF Variants from Second Generation Fab-Phage Libraries*

15

Variants from library YC-81	
Name	VH3 sequence (residues 99-111)
Y0238-1	YPYYRGTSHWYFD (SEQ ID NO:56)
Y0238-2	YPYYINKSHWYFD (SEQ ID NO:57)
20 Y0238-3	YPYYYGTSHWYFD (SEQ ID NO:58)
Y0238-4	YPYYYNQSHWYFD (SEQ ID NO:59)
Y0238-5	YPYYIAKSHWYFD (SEQ ID NO:60)
Y0238-6	YPYYRDNSHWYFD (SEQ ID NO:61)
Y0238-7	YPYYWGTSHWYFD (SEQ ID NO:62)
25 Y0238-8	YPYYRQNSHWYFD (SEQ ID NO:63)
Y0238-9	YPYYRQSSHWYFD (SEQ ID NO:64)
Y0238-10	YPYYRNTSHWYFD (SEQ ID NO:65)
Y0238-11	YPYYKNTSHWYFD (SEQ ID NO:66)
Y0238-12	YPYYIERSHWYFD (SEQ ID NO:67)
30 Y0228-21	YPYYRNASHWYFD (SEQ ID NO:68)
Y0228-22	YPYYTTRSHWYFD (SEQ ID NO:69)
Y0228-23	YPYYEGSSHWYFD (SEQ ID NO:70)

	Y0228-24	YPYYRQRGHWYFD (SEQ ID NO:71)
	Y0228-26	YPYYTGRSHWYFD (SEQ ID NO:72)
	Y0228-27	YPYYTNTSHWYFD (SEQ ID NO:73)
	Y0228-28	YPYYRKGSHWYFD (SEQ ID NO:74)
5	Y0228-29	YPYYTGSSHWYFD (SEQ ID NO:75)
	Y0228-30	YPYYRSGSHWYFD (SEQ ID NO:76)
	Y0229-20	YPYYTNRSHWYFD (SEQ ID NO:77)
	Y0229-21	YPYYRNSSHWYFD (SEQ ID NO:78)
	Y0229-22	YPYYKESSHWYFD (SEQ ID NO:79)
10	Y0229-23	YPYYRDASHWYFD (SEQ ID NO:80)
	Y0229-24	YPYYRQKGHWYFD (SEQ ID NO:81)
	Y0229-25	YPYYKGGSHWYFD (SEQ ID NO:82)
	Y0229-26	YPYYYGASHWYFD (SEQ ID NO:83)
	Y0229-27	YPYYRGESHWYFD (SEQ ID NO:84)
15	Y0229-28	YPYYRSTSHWYFD (SEQ ID NO:85)
<b>Variants from library HL-265</b>		
	<b>Name</b>	<b>VH1 sequence (residue 26-35)</b>
	Y0243-1	GYDFTHYGMN (5/10 clones) (SEQ ID NO:86)
	Y0243-2	GYEFQHYGMN (SEQ ID NO:87)
20	Y0243-3	GYEFTHYGMN (SEQ ID NO:88)
	Y0243-4	GYDFGHYGMN (SEQ ID NO:89)
	Y0243-5	GYDFSHYGMN (SEQ ID NO:90)
	Y0243-6	GYEFSHYGMN (SEQ ID NO:91)
<b>Variants from library YC-101</b>		
25	<b>Name</b>	<b>VH "CDR7" sequence (residues 70-79)</b>
	Y0244-1	FSVDVSKSTA (SEQ ID NO:92)
	Y0244-2	FSLDKSKSTA (SEQ ID NO:93)
	Y0244-3	FSLDVWKSTA (SEQ ID NO:94)
30	Y0244-4	FSIDKSKSTA (:95)



The sequence of the randomized region only is shown as deduced from DNA sequencing.

When a number of clones were tested along with the parent clone pY0192 in phage-ELISA assay, none showed a distinctive improvement over the parental clone. This could  
5 be explained by the time-scale on which the assay was performed (< 3 hours).

In order to quantify improvement in antigen binding over parent clone, several anti-VEGF variants' DNA were transformed into *E. coli* strain 34B8, expressed as Fab, and purified by passing the periplasmic shockate through a protein G column (Pharmacia) as described in Example 2 above.

10 **CDR Combination Variants:** To improve VEGF binding affinity further, mutations found by phage display were combined in different CDRs to create multiple-CDR mutants. In particular, the mutations identified in the most affinity-improved phage variants from VH1, VH2, and VH3 libraries were combined (Table 14) in order to test for additivity of their contributions to binding affinity.

15

**Table 14: Combination CDR Anti-VEGF Variants**

Name	Parent clone	Mutagenesis oligo/ comments	Sequence
Y0313-1	Y0243-1	YC-115 (VH3: H101Y and S105T)	5'-GCAAAGTACCCGTA TACTATTA TGGGACGAGCCACTGGTATTT C-3' (SEQ ID NO:96)
20 Y0317	Y0313-1	YC-108 (revert VL1 back to wild type)	5'-GTCACCATCACCTGCAGCGC AAGTCAGGATATTAGCAACTA TTTAAAC-3' (SEQ ID NO:97)
Y0313-3	Y0238-3	YC-116 (VH3; T105S)	5'-CCGTACTATTATGGGAGCA GCCACTGGTATTTC-3' (SEQ ID NO:98)

25 Mutations from the indicated parental vectors were combined with those from the indicated oligonucleotide by site-directed mutagenesis to yield the combination variants listed.

Version Y0317 is equivalent to Y0313-1 except that the background mutation in VL1 was removed and its sequence reverted back to that in pY0101. The effects of

mutating H101Y and S105T were tested by constructing a reversion mutant from Y0238-3.

**BLAcore Analysis:** The VEGF-binding affinities of Fab fragments were calculated from association and dissociation rate constants measured using a BIAcore-2000™ surface plasmon resonance system (BIAcore, Inc., Piscataway, NJ). A biosensor chip was activated for covalent coupling of VEGF using N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier's (BIAcore, Inc., Piscataway, NJ) instructions. VEGF was buffered exchanged into 20 mM sodium acetate, pH 4.8 and diluted to approximately 50 µg/mL. An aliquot (35 µL) was injected at a flow rate of 2 µL/min to achieve approximately 700-1400 response units (RU) of coupled protein. Finally, 1 M ethanolamine was injected as a blocking agent.

For kinetics measurements, two-fold serial dilutions of Fab were injected in PBS/TWEEN™ buffer (0.05% TWEEN 20™ in phosphate buffered saline) at 25°C at a flow rate of 10 µL/min. On rates and off rates were calculated using standard protocols (Karlsson *et al. J. Immun. Methods* 145:229-240 (1991)). Equilibrium dissociation constants, K<sub>d</sub>'s from surface plasmon resonance (SPR) measurements were calculated as koff/kon. Data are shown in Table 15 below.

20

**Table 15: Kinetics of Fab-VEGF binding from BIAcore™ measurements**

Variant	Kon (10 <sup>4</sup> /M/s)	koff (10 <sup>-4</sup> /s)	Kd (nM)	Kd (wt) / Kd (mut)
Y0244-1	3.4	2.7	8	3.6
Y0244-4	5.2	1.7	3.3	0.9
Y0243-1	6.7	0.45	0.7	4.1
Y0238-3	1.7	≤0.04*	≤0.2*	≥14*
Y0238-7	1.5	≤0.06*	≤0.4*	≥7.3*
Y0238-10	1.6	0.09	0.6	4.8
Y0238-5	0.8	0.08	0.9	3.2
Y0238-1	2.6	0.09	0.4	7.3
Y0313-1	3.5	≤0.054*	≤0.15*	≥20*

30

20

**Table 15: Kinetics of Fab-VEGF binding from BIAcore™ measurements**

Y0313-3	1.2	0.081	0.65	4.5
---------	-----	-------	------	-----

\* The dissociation rate observed probably reflects an upper limit for the true dissociation rate in these experiments, since the off-rate is approaching the limit of detection by BIAcore.

5

The BIAcore™ data in Table 15 show that several variants had improved affinity over Y0192. For example, a CDRH1 variant, Y0243-1, showed 4.1 fold enhanced affinity, arising from mutations T28D and N31H. Variant Y0238-3 showed at least a 14 fold improvement in binding affinity over Y0192. Both CDRH3 mutations contribute to the improved affinity of Y0238-3 because reversion of T105 to S (variant Y0313-3) reduces the affinity of Y0238-3 from 0.15nM to 0.65 nM (see Table 15). The greater affinity enhancement relative to Y0192 was seen for Y0313-1, which contained CDRH3 mutations combined with CDRH1 mutations.

**Cell-Based Assay of VEGF Inhibition:** Several versions of the A4.6.1 anti-VEGF antibody were tested for their ability to antagonize VEGF (recombinant; version 1-165) in induction of the growth of HuVECs (human umbilical vein endothelial cells). The 96-well plates were seeded with 1000 HuVECs per well and fasted in assay medium (F12:DMEM 50:50 supplemented with 1.5% diafiltered fetal bovine serum) for 24 h. The concentration of VEGF used for inducing the cells was determined by first titrating for the amount of VEGF that can stimulate 80% of maximal DNA synthesis. Fresh assay medium containing fixed amounts of VEGF (0.2 nM final concentration), and increasing concentrations of anti-VEGF Fab or Mab were then added. After 40 h of incubation, DNA synthesis was measured by incorporation of tritiated thymidine. Cells were pulsed with 0.5  $\mu$ Ci per well of [3H]-thymidine for 24 h and harvested for counting, using a TopCount gamma counter.

The results (Fig. 11) show that the full-length IgG form of F(ab)-12 was significantly more potent in inhibiting VEGF activity than the Fab form (here, Y0192 was used). However, both variants Y0238-3 and Y0313-1 showed even more potent inhibition of VEGF activity than either the Y0192 Fab or F(ab)-12 Mab. Comparing the Fab forms, variant Y0313-1 appeared >30-fold more potent than the wild-type Fab. It should be noted that the amount of VEGF (0.2 nM) used in this assay is potentially limiting for determination of an accurate IC<sub>50</sub> for the mutant. For example, if the binding affinity (K<sub>d</sub>) of the mutant is in fact < 0.2 nM, the IC<sub>50</sub> in this experiment will appear higher than under conditions of

lower VEGF concentration. The result therefore supports the conclusion that the affinity-improved variant is at least 30-fold improved in affinity for VEGF, and that it effectively blocks VEGF activity *in vitro*. Since the variant Y0317 differs from Y0313-1 only in the reversion of the VL1 sequence to wild-type (Fig. 10A), it is predicted that Y0317 will have  
5 similar activity to Y0313-1.

Variant Y0317 (Fab) and humanized variant F(ab)-12 from Example 1 (full length and Fab) were compared for their ability to inhibit bovine capillary endothelial cell proliferation in response to a near maximally effective concentration of VEGF using the assay described in Example 1. As illustrated in Figure 12, Y0317 was markedly more effective at inhibiting  
10 bovine capillary endothelial cell proliferation than the full length and Fab forms of F(ab)-12 in this assay. The Y0317 affinity matured Fab demonstrated an ED50 value in this assay which was at least about 20 fold lower than F(ab)-12 Fab.

WHAT IS CLAIMED IS:

1. A humanized anti-VEGF antibody which binds human VEGF with a  $K_d$  value of no more than about  $1 \times 10^{-8}M$ .
2. A humanized anti-VEGF antibody which binds human VEGF with a  $K_d$  value of no more than about  $5 \times 10^{-9}M$ .
3. A humanized anti-VEGF antibody which has an ED50 value of no more than about 5nM for inhibiting VEGF-induced proliferation of endothelial cells *in vitro*.
4. A humanized anti-VEGF antibody which inhibits VEGF-induced angiogenesis *in vivo*.
5. The humanized anti-VEGF antibody of claim 4 wherein 5mg/kg of the antibody inhibits at least about 50% of tumor growth in an A673 *in vivo* tumor model.
6. The humanized anti-VEGF antibody of claim 1 having a heavy chain variable domain comprising the following hypervariable region amino acid sequences: CDRH1 (GYX<sub>1</sub>FTX<sub>2</sub>YGMN, wherein X<sub>1</sub> is T or D and X<sub>2</sub> is N or H; SEQ ID NO:128), CDRH2 (WINTYTGEPTYAADFQR; SEQ ID NO:2) and CDRH3 (YPX<sub>1</sub>YYGX<sub>2</sub>SHWYFDV, wherein X<sub>1</sub> is Y or H and X<sub>2</sub> is S or T; SEQ ID NO:129).
7. The humanized anti-VEGF antibody of claim 6 comprising the amino acid sequence of SEQ ID NO:7.
8. The humanized anti-VEGF antibody of claim 6 having a heavy chain variable domain comprising the following hypervariable region amino acid sequences: CDRH1 (GYTFTNYGMN; SEQ ID NO:1), CDRH2 (WINTYTGEPTYAADFQR; SEQ ID NO:2) and CDRH3 (YPHYYGSSHWYFDV; SEQ ID NO:3).

9. The humanized anti-VEGF antibody of claim 1 having a light chain variable domain comprising the following hypervariable region amino acid sequences: CDRL1 (SASQDISNYLN; SEQ ID NO:4), CDRL2 (FTSSLHS; SEQ ID NO:5) and CDRL3 (QQYSTVPWT; SEQ ID NO:6).
- 5
10. The humanized anti-VEGF antibody of claim 9 comprising the amino acid sequence of SEQ ID NO:8.
11. The humanized anti-VEGF antibody of claim 1 having a heavy chain variable domain
- 10 comprising the amino acid sequence of SEQ ID NO:7 and a light chain variable domain comprising the amino acid sequence of SEQ ID NO:8.
12. An anti-VEGF antibody light chain variable domain comprising the amino acid sequence:
- 15 DIQX<sub>1</sub>TQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIIYFTSSLHSGVPSRFSGSGSGTDFTLTISLQPEDFATYYCQQYSTVPWTFGQGGTKVEIKR (SEQ ID NO:124), wherein X<sub>1</sub> is M or L.
13. An anti-VEGF antibody heavy chain variable domain comprising the amino acid
- 20 sequence:
- EVQLVESGGGLVQPGGSLRLSCAASGYX<sub>1</sub>FTX<sub>2</sub>YGMNWVRQAPGKGLEWVGWI  
 NTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYPX<sub>3</sub>YY  
 GX<sub>4</sub>SHWYFDVWGQGTLLTVSS (SEQ ID NO:125), wherein X<sub>1</sub> is T or D; X<sub>2</sub> is N or H; X<sub>3</sub> is Y or H and X<sub>4</sub> is S or T.
- 25
14. A variant of a parent anti-VEGF antibody, wherein said variant binds human VEGF and comprises an amino acid substitution in a hypervariable region of a heavy chain variable domain of said parent antibody.
- 30 15. The variant of claim 14 wherein said parent antibody is a human or humanized antibody.

16. The variant of claim 14 which binds human VEGF with a  $K_d$  value of no more than about  $1 \times 10^{-8}M$ .
17. The variant of claim 14 which binds human VEGF with a  $K_d$  value of no more than  
5 about  $5 \times 10^{-9}M$ .
18. The variant of claim 14 wherein the substitution is in CDRH1 of the heavy chain variable domain.
- 10 19. The variant of claim 14 wherein the substitution is in CDRH3 of the heavy chain variable domain.
20. The variant of claim 14 which has amino acid substitutions in both CDRH1 and CDRH3.
- 15 21. The variant of claim 14 which binds human VEGF with a  $K_d$  value less than that of said parent antibody.
22. The variant of claim 14 which has an ED50 value for inhibiting VEGF-induced  
20 proliferation of endothelial cells *in vitro* which is at least about 10 fold lower than that of said parent antibody.
23. The variant of claim 18 wherein the CDRH1 comprises the amino acid sequence:  
GYDFTHYGMN (SEQ ID NO:126)
- 25 24. The variant of claim 19 wherein the CDRH3 comprises the amino acid sequence:  
YPYYYGTSHWYFDV (SEQ ID NO:127).
25. The variant of claim 14 wherein the heavy chain variable domain comprises the amino  
30 acid sequence of SEQ ID NO:116.

26. The variant of claim 25 further comprising the light chain variable domain amino acid sequence of SEQ ID NO:124.
27. The variant of claim 26 comprising the light chain variable domain amino acid  
5 sequence of SEQ ID NO:115.
28. The humanized anti-VEGF antibody of claim 1 which is a full length antibody.
29. The humanized anti-VEGF antibody of claim 28 which is a human IgG.
- 10 30. The humanized anti-VEGF antibody of claim 1 which is an antibody fragment.
31. The antibody fragment of claim 30 which is a Fab.
- 15 32. A composition comprising the humanized anti-VEGF antibody of claim 1 and a pharmaceutically acceptable carrier.
33. A composition comprising the variant anti-VEGF antibody of claim 14 and a pharmaceutically acceptable carrier.
- 20 34. Isolated nucleic acid encoding the antibody of claim 1.
35. A vector comprising the nucleic acid of claim 34.
- 25 36. A host cell comprising the vector of claim 35.
37. A process of producing a humanized anti-VEGF antibody comprising culturing the host cell of claim 36 so that the nucleic acid is expressed.
- 30 38. The process of claim 37 further comprising recovering the humanized anti-VEGF antibody from the host cell culture.



39. A method for inhibiting VEGF-induced angiogenesis in a mammal comprising administering a therapeutically effective amount of the humanized anti-VEGF antibody of claim 1 to the mammal.
- 5 40. The method of claim 39 wherein the mammal is a human.
41. The method of claim 39 wherein the mammal has a tumor.
42. The method of claim 39 wherein the mammal has a retinal disorder.

A4.6.1    EIQLVQSGPELKQPGETVRISCKASGYTFTNYGMNWKQA  
            \*       \*\* \*        \*\*\* \*     \*

F(ab)-12 EVQLVESGGGLVPGGSLRLSCAASGYTFTNYGMNWVRQA  
   \*     \*\* \*

humIII    EVQLVESGGGLVPGGSLRLSCAASGFTFSSYAMSWVRQA

A4.6.1      PGKGLKWMGWINTYTGEPTYAADFKRRFTFSLETSASTAYL  
              \* \*    \* \*

F(ab)-12   PGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTAYL  
              \*\* \* \* \* \* \*    \* \* \* \* \*

humIII     PGKGLEWVSVISGDGGSTTYADSVKGRFTISRDN SKNTLYL

	abc	90		110	
A4.6.1	QISNLKND	TATYFC	<u>AKYPHYYGSSHWYFDV</u>	WGAGTT	TVTVSS (SEQ.ID NO:9)
	*** **	* *		* *	
F(ab)-12	QMNSLRAED	TA	<u>AKYPHYYGSSHWYFDV</u>	WGQGL	TVTVSS (SEQ.ID NO:7)
			*	*	
humIII	QMNSLRAED	TA	CARG-----	FDYWGQGL	TVTVSS (SEQ.ID NO:11)

A4.6.1    DIQMTQTSSLSASLGDRVIISCSASODISNYLNWYQQKP  
               \*\*                \*            \*   \*

F(ab)-12   DIQMQSPSSLASVGDVRTITCSASQDISNYLNWYQQKP  
   \*                \*

humKI    DIQMOSPSSLASVGDRVTTTCRASOSISNYLAWYOOPK

A4.6.1      DGTVKVLII**YFTSSLHS**GVPSPRFGSGSGSGTDYSLTISNLEP  
              \* \* \* \*                                  \*\*             \* \*

F(ab)-12   GKAPKVLII**YFTSSLHS**GVPSPRFGSGSGSGTDFTLTISLQNP  
              \*        \*\*               \*

humKI      GKAPKLIIYAASSLESGVPSRFSGSGSGTDFTLTISLSLP

	90	100	
A4.6.1	EDIATYYC <u>QQY</u> STVPWTFGGGTKLEIKR		(SEQ.ID NO:10)
	*	* *	
F(ab)-12	EDFATYYC <u>QQY</u> STVPWTFGGQGTKVEIKR		(SEQ.ID NO:8)
		***	
humKI	EDFATYYCQQYNSLPWTFGGQGTKVEIKR		(SEQ.ID NO:12)

**SUBSTITUTE SHEET (RULE 26)**

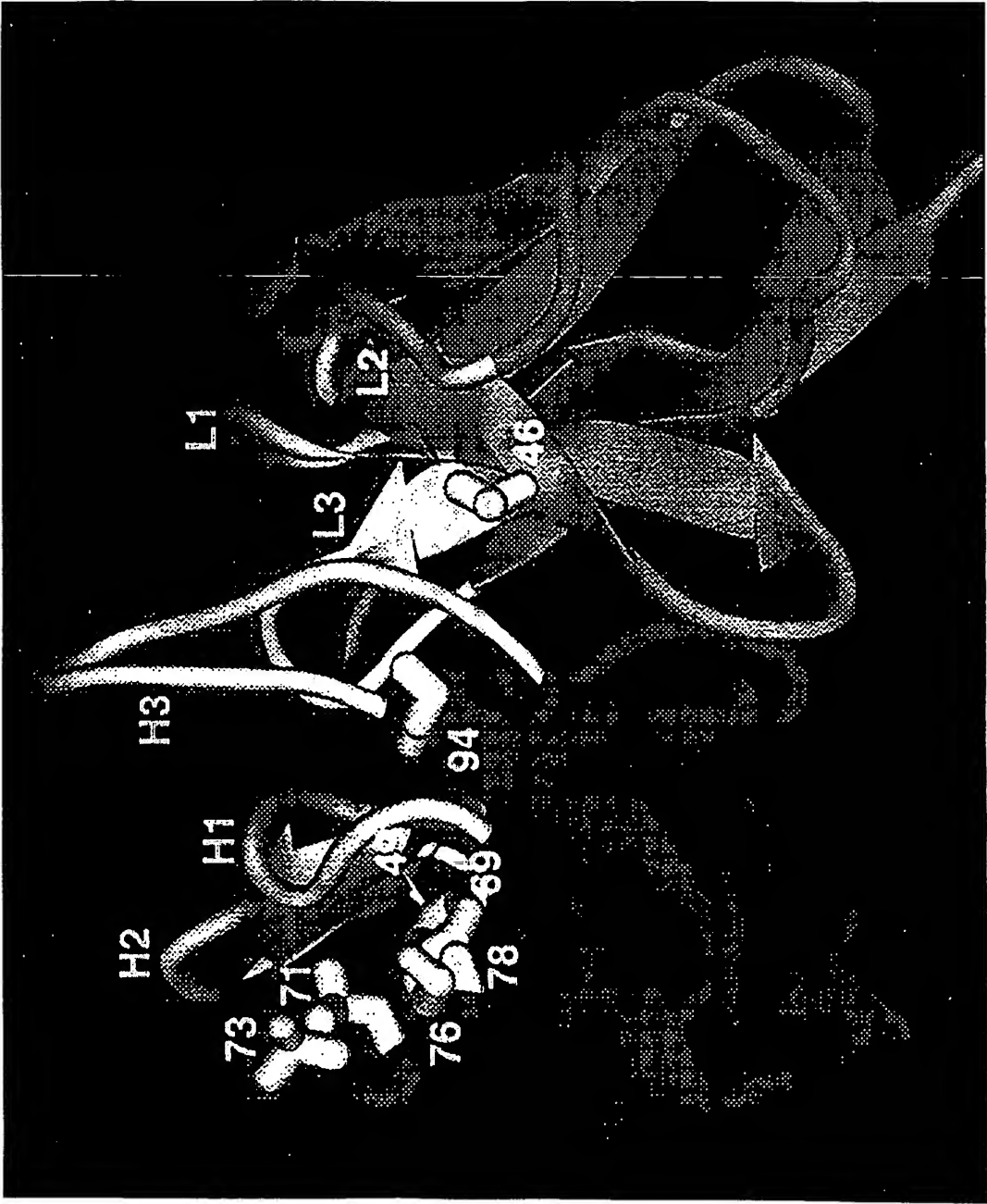
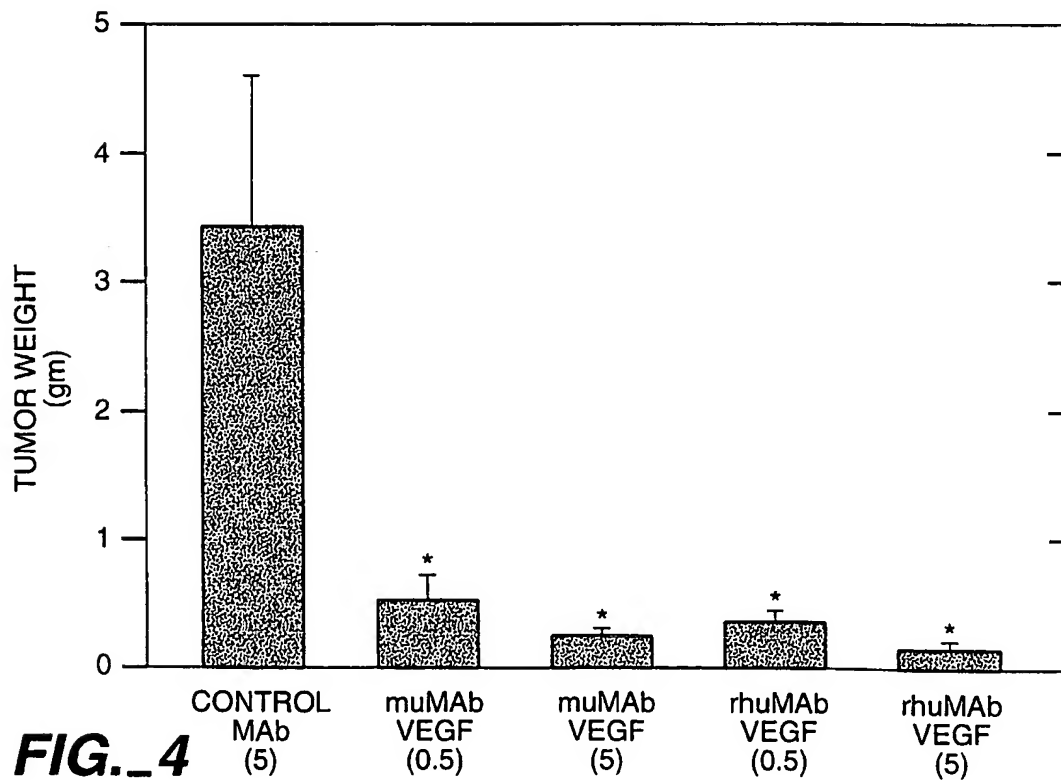
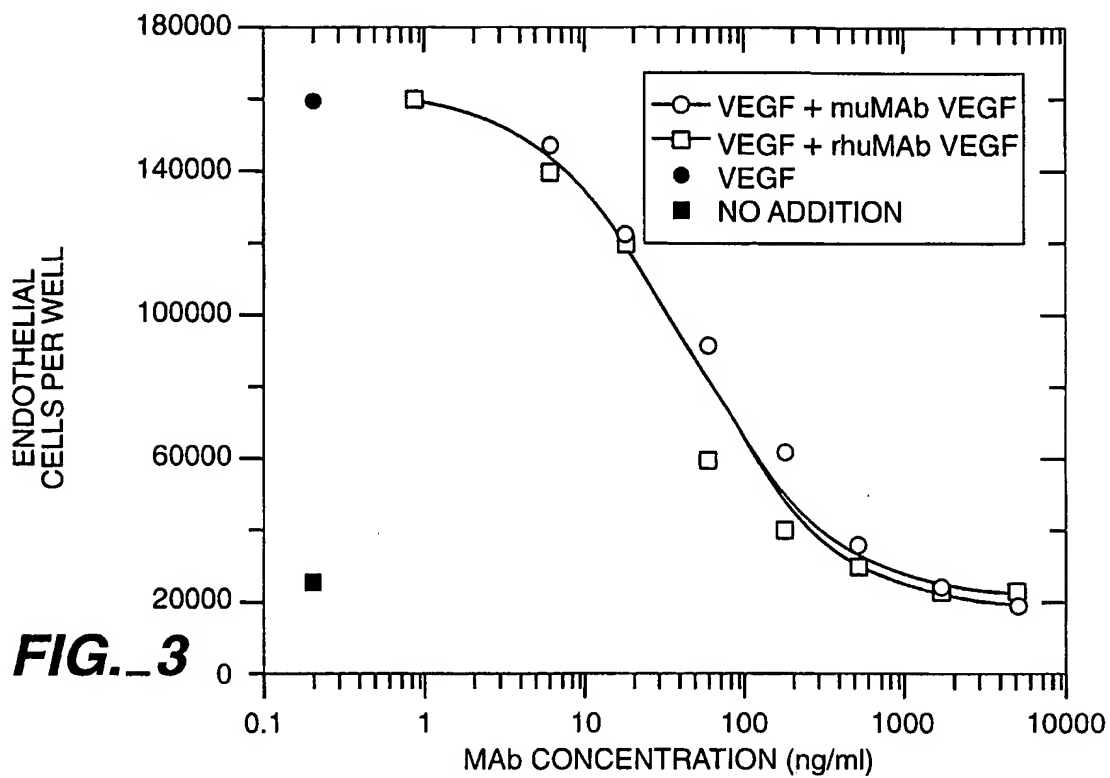


FIG. 2

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	10	20	30	40	
A4.6.1	DIQMTQTTSSLSASLGDRVIISCSASQDISNYLNWYQQKP				
	**	*	* *		
hu2.0	DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKP				
hu2.10	DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKP				

	50	60	70	80	
A4.6.1	DGTVKVLIYFTSSLHSGVPSRFSGSGSGTDYSLTISNLEP				
	**** *		**	* *	
hu2.0	GKAPKLLIYFTSSLHSGVPSRFSGSGSGTDFTLTISSLQP				
hu2.10	GKAPKLLIYFTSSLHSGVPSRFSGSGSGTDYTLTISSLQP				

	90	100	
A4.6.1	EDIATYYCQQYSTVPWTFGGGKLEIK	(SEQ.ID NO:10)	
	*	* *	
hu2.0	EDFATYYCQQYSTVPWTFGQGTKVEIK	(SEQ.ID NO:13)	
hu2.10	EDFATYYCQQYSTVPWTFGQGTKVEIK	(SEQ.ID NO:15)	

**FIG.\_5A**

	10	20	30	40	
A4.6.1	EIQLVQSGPELKQPGETVRI SCKASGYTFTNYGMNWVKQA				
	*	* ** *	*** *	*	
hu2.0	EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQA				
hu2.10	EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWIRQA				

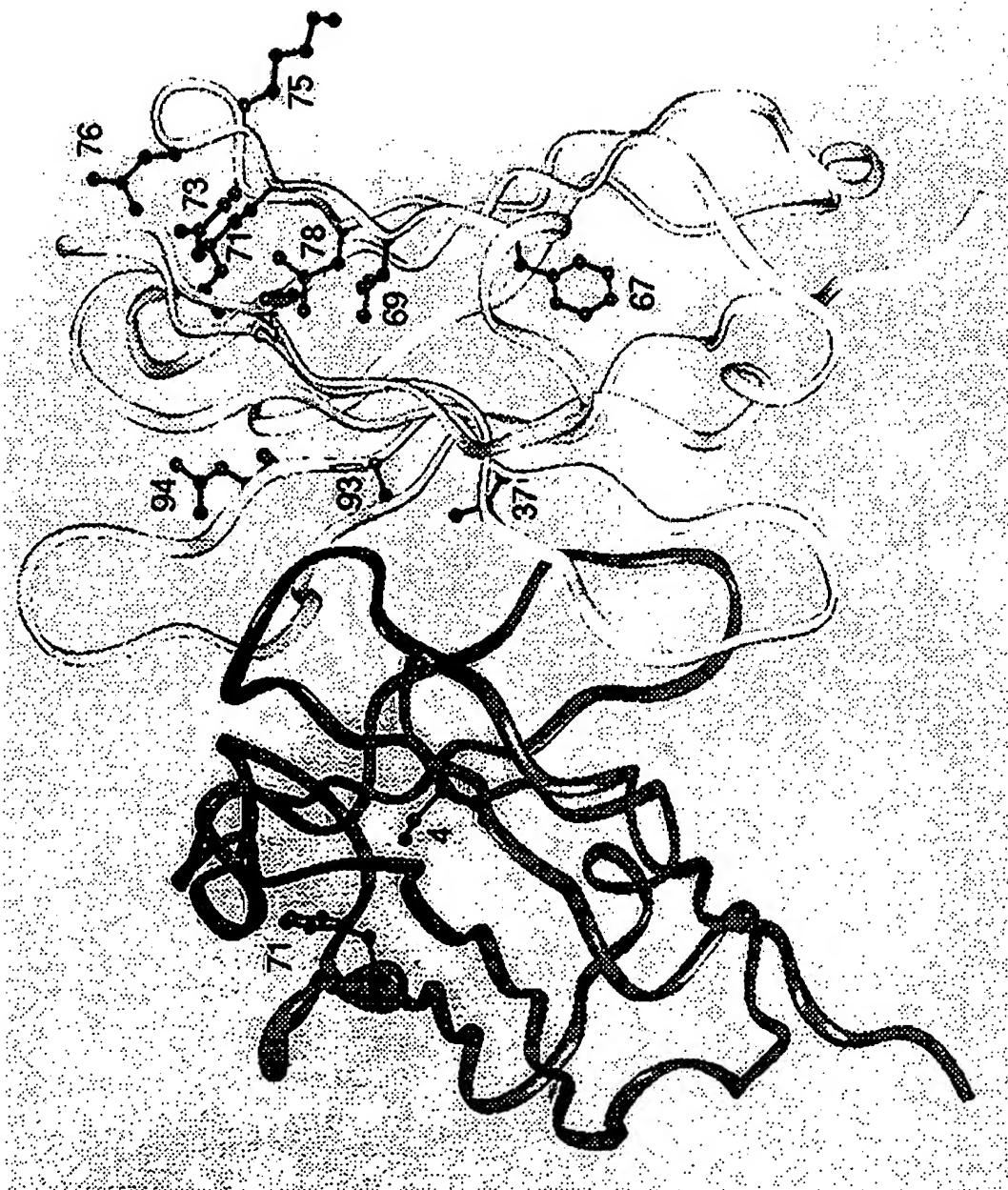
  

	50 a	60	70	80	
A4.6.1	PGKGLKWMGWINTYTGEPTYAADFKRRFTFSLETSASTAYL				
	* *		* *** ** *		
hu2.0	PGKGLEWVGWINTYTGEPTYAADFKRRFTISRDN SKNTLYL				
hu2.10	PGKGLEWVGWINTYTGEPTYAADFKRRFTISLDTSASTVYL				

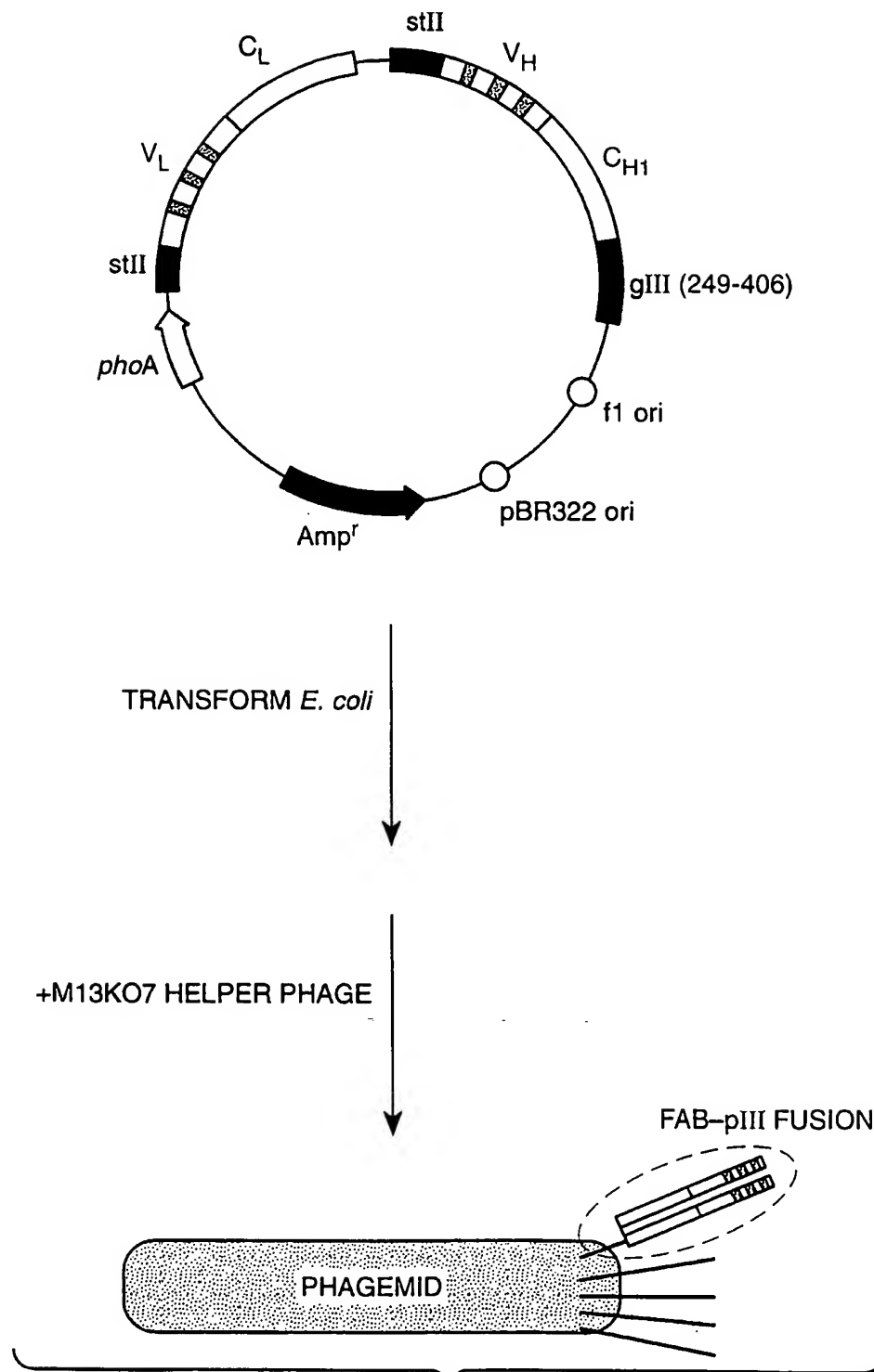
	abc	90	100abcdef	110	
A4.6.1	QISNLKNDDTATYFCAKYPHYYGSSHWFVDVWGAGTTVTVSS	(SEQ.ID NO:9)			
	*** ** *	* *			
hu2.0	QMNSLRAEDTAVYYCARYPHYYGSSHWFVDVWGQGLVTVSS	(SEQ.ID NO:14)			
hu2.10	QMNSLRAEDTAVYYCARYPHYYGSSHWFVDVWGQGLVTVSS	(SEQ.ID NO:16)			

**FIG.\_5B**



**FIG. 6**

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**FIG. 7**

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GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC TCATTGCTGA 60

GTTGTTATTT AAGCTTTGGA GATTATCGTC ACTGCAATGC TTCGCAATAT GGCGCAAAAT 120

GACCAACAGC GGTGATTGA TCAGGTAGAG GGGGCGCTGT ACGAGGTAAA GCCCGATGCC 180

AGCATTCCTG ACGACGATAC GGAGCTGCTG CGCGATTACG TAAAGAAGTT ATTGAAGCAT 240

CCTCGTCAGT AAAAAGTTAA TCTTTTCAAC AGCTGTCATA AAGTTGTCAC GGCCGAGACT 300

TATAGTCGCT TTGTTTTTAT TTTTAAATGT ATTTGTAACT AGAATTCGAG CTCGGTACCC 360

GGGGATCCTC TAGAGGTTGA GGTGATTTT ATG AAA AAG AAT ATC GCA TTT CTT 413  
Met Lys Lys Asn Ile Ala Phe Leu  
-23 -20

CTT GCA TCT ATG TTC GTT TTT TCT ATT GCT ACA AAC GCG TAC GCT GAT 461  
Leu Ala Ser Met Phe Val Phe Ser Ile Ala Thr Asn Ala Tyr Ala Asp  
-15 -10 -5 1

BEGIN stII SIGNAL SEQUENCE

ATC CAG TTG ACC CAG TCC CCG AGC TCC CTG TCC GCC TCT GTG GGC GAT 509  
Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp  
5 10 15

AGG GTC ACC ATC ACC TGC AGC GCA AGT CAG GAT ATT AGC AAC TAT TTA 557  
Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser Asn Tyr Leu  
20 25 30

BEGIN LIGHT CHAIN

AAC TGG TAT CAA CAG AAA CCA GGA AAA GCT CCG AAA CTA CTG ATT TAC 605  
Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr  
35 40 45

TTC ACC TCC TCT CTC CAC TCT GGA GTC CCT TCT CGC TTC TCT GGA TCC 653  
Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly Ser  
50 55 60 65

GGT TCT GGG ACG GAT TAC ACT CTG ACC ATC AGC AGT CTG CAG CCA GAA 701  
Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu  
70 75 80

GAC TTC GCA ACT TAT TAC TGT CAA CAG TAT AGC ACC GTG CCG TGG ACG 749  
Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Thr Val Pro Trp Thr  
85 90 95

TTT GGA CAG GGT ACC AAG GTG GAG ATC AAA CGA ACT GTG GCT GCA CCA 797  
Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro  
100 105 110

TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA ACT 845  
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr  
115 120 125

GCT TCT GTT GTG TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG GCC AAA 893  
Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys  
130 135 140 145

**FIG. 8A**



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GTA Val	CAG Gln	TGG Trp	AAG Lys	GTG Val 150	GAT Asp	AAC Asn	GCC Ala	CTC Leu	CAA Gln 155	TCG Ser	GGT Gly	AAC Asn	TCC Ser	CAG Gln 160	GAG Glu	941
AGT Ser	GTC Val	ACA Thr	GAG Glu 165	CAG Gln	GAC Asp	AGC Ser	AAG Lys	GAC Asp 170	AGC Ser	ACC Thr	TAC Tyr	AGC Ser	CTC Leu 175	AGC Ser	AGC Ser	989
ACC Thr	CTG Leu	ACG Thr 180	CTG Leu	AGC Ser	AAA Lys	GCA Ala	GAC Asp 185	TAC Tyr	GAG Glu	AAA Lys	CAC His	AAA Lys 190	GTC Val	TAC Tyr	GCC Ala	1037
TGC Cys	GAA Glu 195	GTC Val	ACC Thr	CAT His	CAG Gln	GGC Gly 200	CTG Leu	AGC Ser	TCG Ser	CCC Pro	GTC Val 205	ACA Thr	AAG Lys	AGC Ser	TTC Phe	1085
AAC Asn 210	AGG Arg	GGA Gly	GAG Glu	TGT Cys	T O	AAGCTGATCC TCTACGCCGG ACGCATCGTG										1131
GCCCTAGTAC GCAACTAGTC GTAAAAAGGG TATCTAGAGG TTGAGGTGAT TTT ATG Met -23																1187
BEGIN stII SIGNAL SEQUENCE																
AAA Lys	AAG Lys	AAT Asn -20	ATC Ile	GCA Ala	TTT Phe	CTT Leu -15	CTT Leu	GCA Ala	TCT Ser	ATG Met	TTC Phe	GTT Val -10	TTT Phe	TCT Ser	ATT Ile	1235
GCT Ala -5	ACA Thr	AAC Asn	GCG Ala	TAC Tyr	GCT Ala	GAG Glu 1	GTT Val	CAG Gln	CTG Leu	GTG Val 5	GAG Glu	TCT Ser	GGC Gly	GGT Gly 10	GGC Gly	1283
CTG Leu	GTG Val	CAG Gln	CCA Pro	GGG Gly 15	GGC Gly	TCA Ser	CTC Leu	CGT Arg	TTG Leu 20	TCC Ser	TGT Cys	GCA Ala	GCT Ala	TCT Ser 25	GGC Gly	1331
BEGIN HEAVY CHAIN																
TAT Tyr	ACC Thr	TTC Phe	ACC Thr 30	AAC Asn	TAT Tyr	GGT Gly	ATG Met	AAC Asn 35	TGG Trp	ATC Ile	CGT Arg	CAG Gln	GCC Ala 40	CCG Pro	GGT Gly	1379
AAG Lys	GGC Gly	CTG Leu 45	GAA Glu	TGG Trp	GTT Val	GGA Gly	TGG Trp 50	ATT Ile	AAC Asn	ACC Thr	TAT Tyr	ACC Thr 55	GGT Gly	GAA Glu	CCG Pro	1427
ACC Thr	TAT Tyr 60	GCT Ala	GCG Ala	GAT Asp	TTC Phe	AAA Lys 65	CGT Arg	CGT Arg	TTT Phe	ACT Thr	ATA Ile 70	TCT Ser	GCA Ala	GAC Asp	ACC Thr	1475
TCC Ser 75	AGC Ser	AAC Asn	ACA Thr	GTT Val	TAC Tyr 80	CTG Leu	CAG Gln	ATG Met	AAC Asn	AGC Ser 85	CTG Leu	CGC Arg	GCT Ala	GAG Glu 90	GAC Asp	1523
ACT Thr	GCC Ala	GTC Val	TAT Tyr	TAC Tyr 95	TGT Cys	GCA Ala	AAG Lys	TAC Tyr	CCG Pro 100	CAC His	TAT Tyr	TAT Tyr	GGG Gly 105	AGC Ser	AGC Ser	1571

**FIG. 8B**

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CAC	TGG	TAT	TTC	GAC	GTC	TGG	GGT	CAA	GGA	ACC	CTG	GTC	ACC	GTC	TCC	1619
His	Trp	Tyr	Phe	Asp	Val	Trp	Gly	Gln	Gly	Thr	Leu	Val	Thr	Val	Ser	
			110					115					120			
TCG	GCC	TCC	ACC	AAG	GGC	CCA	TCG	GTC	TTC	CCC	CTG	GCA	CCC	TCC	TCC	1667
Ser	Ala	Ser	Thr	Lys	Gly	Pro	Ser	Val	Phe	Pro	Leu	Ala	Pro	Ser	Ser	
			125				130					135				
AAG	AGC	ACC	TCT	GGG	GGC	ACA	GCG	GCC	CTG	GGC	TGC	CTG	GTC	AAG	GAC	1715
Lys	Ser	Thr	Ser	Gly	Gly	Thr	Ala	Ala	Leu	Gly	Cys	Leu	Val	Lys	Asp	
			140				145				150					
TAC	TTC	CCC	GAA	CCG	GTG	ACG	GTG	TCG	TGG	AAC	TCA	GGC	GCC	CTG	ACC	1763
Tyr	Phe	Pro	Glu	Pro	Val	Thr	Val	Ser	Trp	Asn	Ser	Gly	Ala	Leu	Thr	
					160					165					170	
AGC	GGC	GTG	CAC	ACC	TTC	CCG	GCT	GTC	CTA	CAG	TCC	TCA	GGA	CTC	TAC	1811
Ser	Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu	Gln	Ser	Ser	Gly	Leu	Tyr	
				175					180					185		
TCC	CTC	AGC	AGC	GTG	GTG	ACC	GTG	CCC	TCC	AGC	AGC	TTG	GGC	ACC	CAG	1859
Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser	Ser	Ser	Leu	Gly	Thr	Gln	
				190				195					200			
ACC	TAC	ATC	TGC	AAC	GTG	AAT	CAC	AAG	CCC	AGC	AAC	ACC	AAG	GTC	GAC	1907
Thr	Tyr	Ile	Cys	Asn	Val	Asn	His	Lys	Pro	Ser	Asn	Thr	Lys	Val	Asp	
			205				210					215				
AAG	AAA	GTT	GAG	CCC	AAA	TCT	TGT	GAC	AAA	ACT	CAC	CTC	TAG	AGT	GGC	1955
Lys	Lys	Val	Glu	Pro	Lys	Ser	Cys	Asp	Lys	Thr	His	Leu	*	Ser	Gly	
			220				225				230					
GGT	GGC	TCT	GGT	TCC	GGT	GAT	TTT	GAT	TAT	GAA	AAG	ATG	GCA	AAC	GCT	2003
Gly	Gly	Ser	Gly	Ser	Gly	Asp	Phe	Asp	Tyr	Glu	Lys	Met	Ala	Asn	Ala	
					240					245					250	
										END LIGHT CHAIN					BE	
AAT	AAG	GGG	GCT	ATG	ACC	GAA	AAT	GCC	GAT	GAA	AAC	GCG	CTA	CAG	TCT	2051
Asn	Lys	Gly	Ala	Met	Thr	Glu	Asn	Ala	Asp	Glu	Asn	Ala	Leu	Gln	Ser	
				255					260					265		
GIN g3p DOMAIN																
GAC	GCT	AAA	GGC	AAA	CTT	GAT	TCT	GTC	GCT	ACT	GAT	TAC	GGT	GCT	GCT	2099
Asp	Ala	Lys	Gly	Lys	Leu	Asp	Ser	Val	Ala	Thr	Asp	Tyr	Gly	Ala	Ala	
			270					275					280			
ATC	GAT	GGT	TTC	ATT	GGT	GAC	GTT	TCC	GGC	CTT	GCT	AAT	GGT	AAT	GGT	2147
Ile	Asp	Gly	Phe	Ile	Gly	Asp	Val	Ser	Gly	Leu	Ala	Asn	Gly	Asn	Gly	
			285				290						295			
GCT	ACT	GGT	GAT	TTT	GCT	GGC	TCT	AAT	TCC	CAA	ATG	GCT	CAA	GTC	GGT	2195
Ala	Thr	Gly	Asp	Phe	Ala	Gly	Ser	Asn	Ser	Gln	Met	Ala	Gln	Val	Gly	
			300			305				310						
GAC	GGT	GAT	AAT	TCA	CCT	TTA	ATG	AAT	AAT	TTC	CGT	CAA	TAT	TTA	CCT	2243
Asp	Gly	Asp	Asn	Ser	Pro	Leu	Met	Asn	Asn	Phe	Arg	Gln	Tyr	Leu	Pro	
					320					325					330	

**FIG.\_8C**

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TCC CTC CCT CAA TCG GTT GAA TGT CGC CCT TTT GTC TTT AGC GCT GGT	2291
Ser Leu Pro Gln Ser Val Glu Cys Arg Pro Phe Val Phe Ser Ala Gly	
335 340 345	
AAA CCA TAT GAA TTT TCT ATT GAT TGT GAC AAA ATA AAC TTA TTC CGT	2339
Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys Ile Asn Leu Phe Arg	
350 355 360	
GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT ATG TAT GTA TTT	2387
Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val Phe	
365 370 375	
TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT TAATCATGCC	2433
Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser OC* (SEQ.ID NO:100)	
380 385 390	
AGTTCTTTTG GCTAGCGCCG CCCTATACCT TGTCTGCCTC CCCGCGTTGC GTCGCGGTGC	2493
END G3 PROTEIN	
ATGGAGCCGG GCCACCTCGA CCTGAATGGA AGCCGGCGGC ACCTCGCTAA CGGATTCACC	2553
ACTCCAAGAA TTGGAGCCAA TCAATTCTTG CGGAGAACTG TGAATGCGCA AACCAACCCT	2613
TGGCAGAACA TATCCATCGC GTCCGCCATC TCCAGCAGCC GCACGCGGCG CATCTCGGGC	2673
AGCGTTGGGT CCTGGCCACG GGTGCGCATG ATCGTGCTCC TGTCGTTGAG GACCCGGCTA	2733
GGCTGGCGGG GTTGCCCTTAC TGGTTAGCAG AATGAATCAC CGATACGCGA GCGAACGTGA	2793
AGCGACTGCT GCTGCAAAAC GTCTGCGACC TGAGCAACAA CATGAATGGT CTTCGGTTTC	2853
CGTGTTTCGT AAAGTCTGGA AACGCGGAAG TCAGCGCCCT GCACCATTAT GTTCCGGATC	2913
TGCATCGCAG GATGCTGCTG GCTACCCTGT GGAACACCTA CATCTGTATT AACGAAGCGC	2973
TGGCATTGAC CCTGAGTGAT TTTTCTCTGG TCCCGCCGCA TCCATACCGC CAGTTGTTTA	3033
CCCTCACAAAC GTTCCAGTAA CCGGGCATGT TCATCATCAG TAACCCGTAT CGTGAGCATC	3093
CTCTCTCGTT TCATCGGTAT CATTACCCCC ATGAACAGAA ATTCCCCCTT ACACGGAGGC	3153
ATCAAGTGAC CAAACAGGAA AAAACCGCCC TTAACATGGC CCGCTTTATC AGAAGCCAGA	3213
CATTAACGCT TCTGGAGAAA CTCAACGAGC TGGACGCGGA TGAACAGGCA GACATCTGTG	3273
AATCGCTTCA CGACCACGCT GATGAGCTTT ACCGCAGGAT CCGGAAATTG TAAACGTTAA	3333
TATTTTGTTA AAATTCGCGT TAAATTTTGG TTAAATCAGC TCATTTTTTA ACCAATAGGC	3393
CGAAATCGGC AAAATCCCTT ATAAATCAAA AGAATAGACC GAGATAGGGT TGAGTGTTGT	3453
TCCAGTTTGG AACAAGAGTC CACTATTAAA GAACGTGGAC TCCAACGTCA AAGGGCGAAA	3513
AACCGTCTAT CAGGGCTATG GCCCACTACG TGAACCATCA CCCTAATCAA GTTTTTTGGG	3573
GTCGAGGTGC CGTAAAGCAC TAAATCGGAA CCCTAAAGGG AGCCCCGAT TTAGAGCTTG	3633

**FIG.\_8D**

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ACGGGGAAAG	CCGGCGAACG	TGGCGAGAAA	GGAAGGGAAG	AAAGCGAAAG	GAGCGGGCGC	3693
TAGGGCGCTG	GCAAGTGTAG	CGGTCACGCT	GCGCGTAACC	ACCACACCCG	CCGCGCTTAA	3753
TGCGCCGCTA	CAGGGCGCGT	CCGGATCCTG	CCTCGCGCGT	TTCGGTGATG	ACGGTGAAAA	3813
CCTCTGACAC	ATGCAGCTCC	CGGAGACGGT	CACAGCTTGT	CTGTAAGCGG	ATGCCGGGAG	3873
CAGACAAGCC	CGTCAGGGCG	CGTCAGCGGG	TGTTGGCGGG	TGTCGGGGCG	CAGCCATGAC	3933
CCAGTCACGT	AGCGATAGCG	GAGTGTATAC	TGGCTTAACT	ATGCGGCATC	AGAGCAGATT	3993
GTACTGAGAG	TGCACCATAT	GCGGTGTGAA	ATACCGCACA	GATGCGTAAG	GAGAAAATAC	4053
CGCATCAGGC	GCTCTTCCGC	TTCCTCGCTC	ACTGACTCGC	TGCGCTCGGT	CGTTCGGCTG	4113
CGGCGAGCGG	TATCAGCTCA	CTCAAAGGCG	GTAATACGGT	TATCCACAGA	ATCAGGGGAT	4173
AACGCAGGAA	AGAACATGTG	AGCAAAAGGC	CAGCAAAAGG	CCAGGAACCG	TAAAAAGGCC	4233
GCGTTGCTGG	CGTTTTTCCA	TAGGCTCCGC	CCCCCTGACG	AGCATCACAA	AAATCGACGC	4293
TCAAGTCAGA	GGTGGCGAAA	CCCGACAGGA	CTATAAAGAT	ACCAGGCGTT	TCCCCCTGGA	4353
AGCTCCCTCG	TGCGCTCTCC	TGTTCCGACC	CTGCCGCTTA	CCGATACCT	GTCCGCCTTT	4413
CTCCCTTCGG	GAAGCGTGGC	GCTTTCTCAT	AGCTCACGCT	GTAGGTATCT	CAGTTCGGTG	4473
TAGGTCGTTT	GCTCCAAGCT	GGGCTGTGTG	CACGAACCCC	CCGTTTACCC	CGACCGCTGC	4533
GCCTTATCCG	GTAACATATC	TCTTGAGTCC	AACCCGGTAA	GACACGACTT	ATCGCCACTG	4593
GCAGCAGCCA	CTGGTAACAG	GATTAGCAGA	GCGAGGTATG	TAGGCGGTGC	TACAGAGTTC	4653
TTGAAGTGGT	GGCCTAACTA	CGGCTACACT	AGAAGGACAG	TATTTGGTAT	CTGCGCTCTG	4713
CTGAAGCCAG	TTACCTTCGG	AAAAAGAGTT	GGTAGCTCTT	GATCCGGCAA	ACAAACCACC	4773
GCTGGTAGCG	GTGGTTTTTT	TGTTTGCAAG	CAGCAGATTA	CGCGCAGAAA	AAAAGGATCT	4833
CAAGAAGATC	CTTTGATCTT	TTCTACGGGG	TCTGACGCTC	AGTGGAACGA	AAACTCACGT	4893
TAAGGGATTT	TGGTCATGAG	ATTATCAAAA	AGGATCTTCA	CCTAGATCCT	TTTAAATTAA	4953
AAATGAAGTT	TTAAATCAAT	CTAAAGTATA	TATGAGTAAA	CTTGGTCTGA	CAGTTACCAA	5013
TGCTTAATCA	GTGAGGCACC	TATCTCAGCG	ATCTGTCTAT	TTGTTTCATC	CATAGTTGCC	5073
TGACTCCCCG	TCGTGTAGAT	AACTACGATA	CGGGAGGGCT	TACCATCTGG	CCCCAGTGCT	5133
GCAATGATAC	CGCGAGACCC	ACGCTCACCG	GCTCCAGATT	TATCAGCAAT	AAACCAGCCA	5193
GCCGGAAGGG	CCGAGCGCAG	AAGTGGTCCT	GCAACTTTAT	CCGCCTCCAT	CCAGTCTATT	5253

**FIG. 8E**

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AATTGTTGCC GGAAGCTAG AGTAAGTAGT TCGCCAGTTA ATAGTTTGCG CAACGTTGTT 5313  
GCCATTGCTG CAGGCATCGT GGTGTCACGC TCGTCGTTTG GTATGGCTTC ATTCAGCTCC 5373  
GGTCCCAAC GATCAAGGCG AGTTACATGA TCCCCATGT TGTGCAAAAA AGCGGTTAGC 5433  
TCCTTCGGTC CTCCGATCGT TGTCAGAAGT AAGTTGGCCG CAGTGTTATC ACTCATGGTT 5493  
ATGGCAGCAC TGCATAATTC TCTTACTGTC ATGCCATCCG TAAGATGCTT TTCTGTGACT 5553  
GGTGAGTACT CAACCAAGTC ATTCTGAGAA TAGTGTATGC GCGACCGAG TTGCTCTTGC 5613  
CCGGCGTCAA CACGGGATAA TACCGCGCCA CATAGCAGAA CTTTAAAAGT GCTCATCATT 5673  
GGAAAACGTT CTTCGGGGCG AAAACTCTCA AGGATCTTAC CGCTGTTGAG ATCCAGTTCC 5733  
ATGTAACCCA CTCGTGCACC CAACTGATCT TCAGCATCTT TTACTTTCAC CAGCGTTTCT 5793  
GGGTGAGCAA AAACAGGAAG GCAAAATGCC GCAAAAAAGG GAATAAGGGC GACACGGAAA 5853  
TGTTGAATAC TCATACTCTT CCTTTTTCAA TATTATTGAA GCATTTATCA GGGTTATTGT 5913  
CTCATGAGCG GATACATATT TGAATGTATT TAGAAAAATA AACAAATAGG GGTCCGCGC 5973  
ACATTTCCCC GAAAAGTGCC ACCTGACGTC TAAGAAACCA TTATTATCAT GACATTAACC 6033  
TATAAAAATA GCGTATCAC GAGGCCCTTT CGTCTTCAA 6072

**FIG.\_8F**

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	10	20	30	40
F(ab)-12	DIQMTQSPSS	LSASVGDRVT	ITCSASQDIS	NYLNWYQQKP
MB1.6	DIQMTQSPSS	LSASVGDRVT	ITCSASQDIS	NYLNWYQQKP
H2305.6	DIQMTQSPSS	LSASVGDRVT	ITCSASQDIS	NYLNWYQQKP
Y0101	DIQMTQSPSS	LSASVGDRVT	ITCSASQDIS	NYLNWYQQKP
Y0192	DIQMTQSPSS	LSASVGDRVT	ITCRANEQLS	NYLNWYQQKP
CDR-L1				
	50	60	70	80
F(ab)-12	GKAPKVLIIYF	TSSLHSGVPS	RFSGSGSGTD	FTLTISSLQP
MB1.6	GKAPKVLIIYF	TSSLHSGVPS	RFSGSGSGTD	FTLTISSLQP
H2305.6	GKAPKVLIIYF	TSSLHSGVPS	RFSGSGSGTD	FTLTISSLQP
Y0101	GKAPKVLIIYF	TSSLHSGVPS	RFSGSGSGTD	FTLTISSLQP
Y0192	GKAPKVLIIYF	TSSLHSGVPS	RFSGSGSGTD	FTLTISSLQP
CDR-L2				
	90	100	110	
F(ab)-12	EDFATYYCQQ	YSTVPWTFGQ	GTKVEIKRTV	(SEQ.ID NO:8)
MB1.6	EDFATYYCQQ	YSTVPWTFGQ	GTKVEIKRTV	(SEQ.ID NO:101)
H2305.6	EDFATYYCQQ	YSTVPWTFGQ	GTKVEIKRTV	(SEQ.ID NO:103)
Y0101	EDFATYYCQQ	YSTVPWTFGQ	GTKVEIKRTV	(SEQ.ID NO:105)
Y0192	EDFATYYCQQ	YSTVPWTFGQ	GTKVEIKRTV	(SEQ.ID NO:107)
CDR-L3				

FIG. 9A

	10	20	30	40
F(ab)-12	EVQLVESGGG	LVQPGGSLRL	SCAASGYTFT	NYGMNWVRQA
MB1.6	EVQLVESGGG	LVQPGGSLRL	SCAASGYTFT	NYGMNWVRQA
H2305.6	EVQLVESGGG	LVQPGGSLRL	SCAASGYTFT	NYGMNWVRQA
Y0101	EVQLVESGGG	LVQPGGSLRL	SCAASGYTFT	NYGMNWVRQA
Y0192	EVQLVESGGG	LVQPGGSLRL	SCAASGYTFT	NYGMNWVRQA
CDR-H1				
	50	60	70	80
F(ab)-12	PGKGLEWVGW	INTYTGEPTY	AADFRRFTF	SLDTSKSTAY
MB1.6	PGKGLEWVGW	INTYTGEPTY	AADFRRFTF	SLDTSKSTAY
H2305.6	PGKGLEWVGW	INTYTGEPTY	AADFRRFTF	SLDTSKSTAY
Y0101	PGKGLEWVGW	INTYTGEPTY	AADFRRFTF	SLDTSKSTAY
Y0192	PGKGLEWVGW	INTYTGEPTY	AADFRRFTF	SLDTSKSTAY
CDR-H2				
	90	100	110	
F(ab)-12	LQMNSLRAED	TAVYYCAKYP	HYGSSHWFY	DVWGQGT
MB1.6	LQMNSLRAED	TAVYYCAKYP	HYGSSHWFY	DVWGQGT
H2305.6	LQMNSLRAED	TAVYYCAKYP	HYGSSHWFY	DVWGQGT
Y0101	LQMNSLRAED	TAVYYCAKYP	HYGSSHWFY	DVWGQGT
Y0192	LQMNSLRAED	TAVYYCAKYP	HYGSSHWFY	DVWGQGT
CDR-H3				

FIG. 9B

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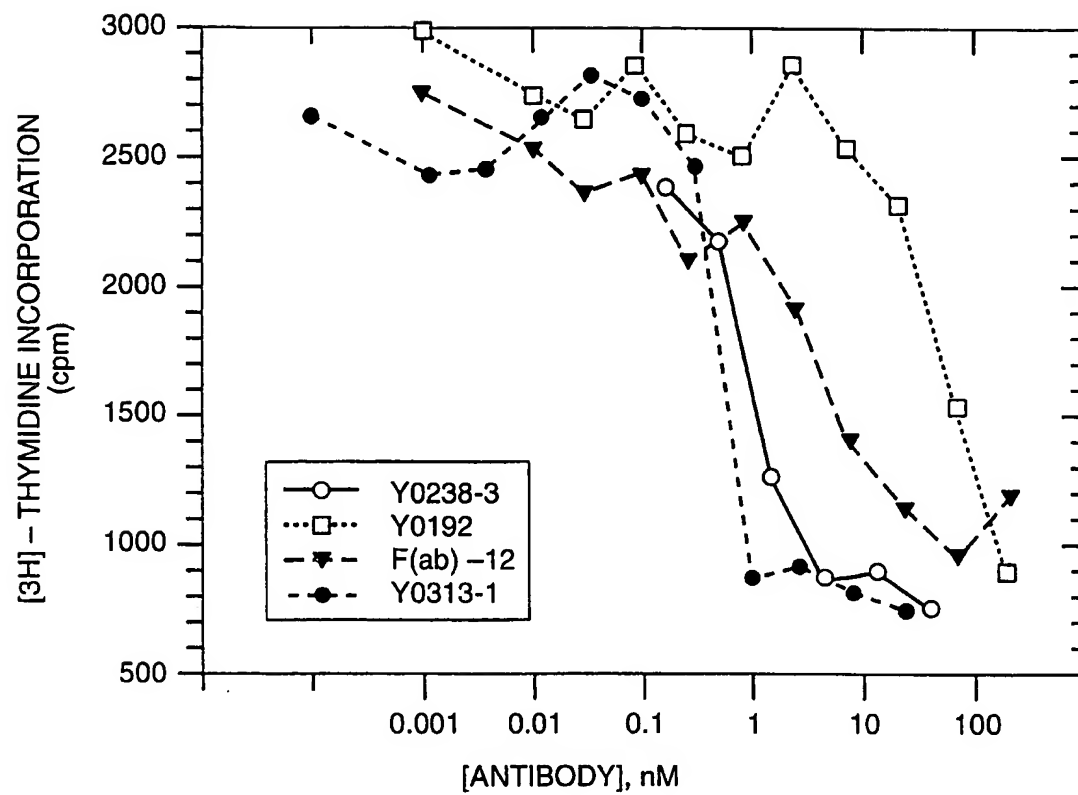
	10	20	30	40
F(ab)-12	DIQMTQSPSS	LSASVGDRVT	ITCSASQDIS	NYLNWYQQKP
Y0243-1	DIQMTQSPSS	LSASVGDRVT	ITCRANEQLS	NYLNWYQQKP
Y0238-3	DIQMTQSPSS	LSASVGDRVT	ITCRANEQLS	NYLNWYQQKP
Y0313-1	DIQMTQSPSS	LSASVGDRVT	ITCRANEQLS	NYLNWYQQKP
Y0317	DIQMTQSPSS	LSASVGDRVT	ITCSASQDIS	NYLNWYQQKP
CDR-L1				
	50	60	70	80
F(ab)-12	GKAPKVLIIYF	TSSLHSGVPS	RFSGSGSGTD	FTLTISSLQP
Y0243-1	GKAPKVLIIYF	TSSLHSGVPS	RFSGSGSGTD	FTLTISSLQP
Y0238-3	GKAPKVLIIYF	TSSLHSGVPS	RFSGSGSGTD	FTLTISSLQP
Y0313-1	GKAPKVLIIYF	TSSLHSGVPS	RFSGSGSGTD	FTLTISSLQP
Y0317	GKAPKVLIIYF	TSSLHSGVPS	RFSGSGSGTD	FTLTISSLQP
CDR-L2				
	90	100	110	
F(ab)-12	EDFATYYCQQ	YSTVPWTFGQ	GTKVEIKRTV	(SEQ.ID NO:8)
Y0243-1	EDFATYYCQQ	YSTVPWTFGQ	GTKVEIKRTV	(SEQ.ID NO:109)
Y0238-3	EDFATYYCQQ	YSTVPWTFGQ	GTKVEIKRTV	(SEQ.ID NO:111)
Y0313-1	EDFATYYCQQ	YSTVPWTFGQ	GTKVEIKRTV	(SEQ.ID NO:113)
Y0317	EDFATYYCQQ	YSTVPWTFGQ	GTKVEIKRTV	(SEQ.ID NO:115)
CDR-L3				

FIG. 10A

	10	20	30	40
F(ab)-12	EVQLVESGGG	LVQPGGSLRL	SCAASGYTFT	NYGMNWVRQA
Y0243-1	EVQLVESGGG	LVQPGGSLRL	SCAASGYDFT	HYGMNWVRQA
Y0238-3	EVQLVESGGG	LVQPGGSLRL	SCAASGYTFT	NYGMNWVRQA
Y0313-1	EVQLVESGGG	LVQPGGSLRL	SCAASGYDFT	HYGMNWVRQA
Y0317	EVQLVESGGG	LVQPGGSLRL	SCAASGYDFT	HYGMNWVRQA
CDR-H1				
	50	60	70	80
F(ab)-12	PGKGLEWVGW	INTYTGEPTY	AADFRRRTF	SLDTSKSTAY
Y0243-1	PGKGLEWVGW	INTYTGEPTY	AADFRRRTF	SLDTSKSTAY
Y0238-3	PGKGLEWVGW	INTYTGEPTY	AADFRRRTF	SLDTSKSTAY
Y0313-1	PGKGLEWVGW	INTYTGEPTY	AADFRRRTF	SLDTSKSTAY
Y0317	PGKGLEWVGW	INTYTGEPTY	AADFRRRTF	SLDTSKSTAY
CDR-H2				
	90	100	110	
F(ab)-12	LQMNSLRAED	TAVYYCAKYP	HYYGSSHWF	DVWGQGT
Y0243-1	LQMNSLRAED	TAVYYCAKYP	HYYGSSHWF	DVWGQGT
Y0238-3	LQMNSLRAED	TAVYYCAKYP	HYYGSSHWF	DVWGQGT
Y0313-1	LQMNSLRAED	TAVYYCAKYP	HYYGSSHWF	DVWGQGT
Y0317	LQMNSLRAED	TAVYYCAKYP	HYYGSSHWF	DVWGQGT
CDR-H3				
CDR-7				

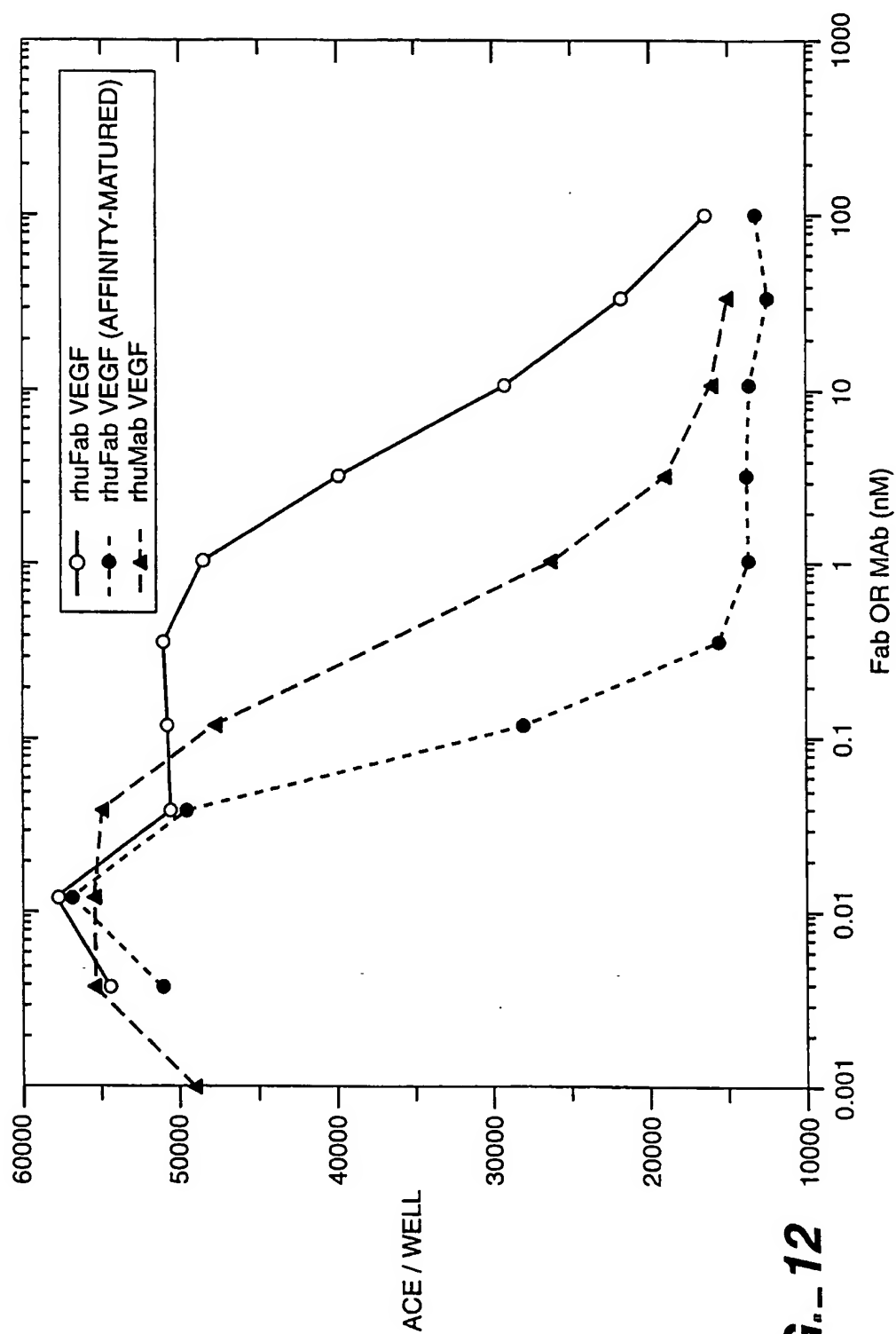
FIG. 10B

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**FIG. 11**



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**FIG. 12**



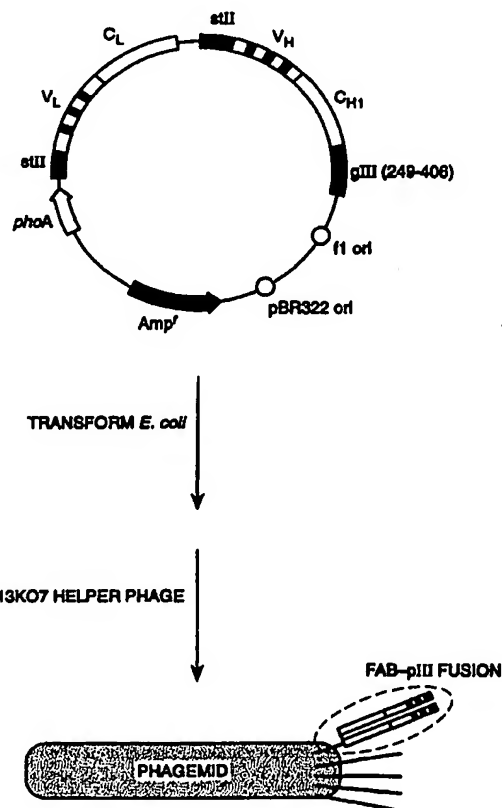
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : C07K 16/22, C12N 15/13, 15/63, 15/70, A61K 39/395	A3	(11) International Publication Number: <b>WO 98/45331</b> (43) International Publication Date: 15 October 1998 (15.10.98)
(21) International Application Number: PCT/US98/06604 (22) International Filing Date: 3 April 1998 (03.04.98) (30) Priority Data: 08/833,504           7 April 1997 (07.04.97)       US 08/908,469           6 August 1997 (06.08.97)       US (71) Applicant (for all designated States except US): GENENTECH, INC. [US/US]; One DNA Way, South San Francisco, CA 94080 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): BACA, Manuel [AU/US]; Apartment #H3, 888 Foster City Boulevard, Foster City, CA 94404 (US). WELLS, James, A. [US/US]; 1341 Columbus Avenue, Burlingame, CA 94010 (US). PRESTA, Leonard, G. [US/US]; Apartment 206, 1900 Gough Street, San Francisco, CA 94109 (US). LOWMAN, Henry, B. [US/US]; 400 San Juan Avenue, El Granada, CA 94018 (US). CHEN, Yvonne, Man-Yee [CA/US]; 1951 O'Farrell Street #321, San Mateo, CA 94403 (US). (74) Agents: DREGER, Walter, H. et al.; Flehr, Hohbach, Test, Albritton & Herbert LLP, Suite 400, 4 Embarcadero Center, San Francisco, CA 94111-4187 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report. (88) Date of publication of the international search report: 3 December 1998 (03.12.98)

(54) Title: ANTI-VEGF ANTIBODIES

## (57) Abstract

Humanized and variant anti-VEGF antibodies and various uses therefor are disclosed. The anti-VEGF antibodies have strong binding affinities for VEGF; inhibit VEGF-induced proliferation of endothelial cells *in vitro*, and inhibit tumor growth *in vivo*.



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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/06604

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K16/22 C12N15/13 C12N15/63 C12N15/70 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 22653 A (GENENTECH INC) 23 December 1992	7, 10-13, 25-27
Y	the whole document and specially: see SEQ.ID.N. 17, 18 and 25 see page 5, line 24 - page 7, line 35  see page 9, line 22 - page 10, line 4; figure 5	1, 4, 14, 15, 22, 28-31, 34-38
Y	KIM ET AL.,: "Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor growth in vivo" NATURE, vol. 362, 1993, page 841 XP002013864 London, GB cited in the application see abstract	1, 4, 14, 15, 22, 28-31, 34-38



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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Date of the actual completion of the international search

18 September 1998

Date of mailing of the international search report

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Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Mateo Rosell, A.M.

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/06604

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GB 2 268 744 A (CELLTECH LTD) 19 January 1994 see abstract see page 4, paragraph 3 - page 6, paragraph 1 ---	1,32-41
A	M.M. BENDIG: "Humanization of rodent monoclonal antibodies" METHODS: A COMPANION TO METHODS IN ENZYMولوجY, vol. 8, 1995, pages 83-93, XP000647344 New York, NY, US see the whole document ---	1,34-38
P,X	M. BACA ET AL., : "Antibody humanization using monovalent phage display" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 16, 18 April 1997, pages 10678-10684, XP002077471 see the whole document -----	1-42

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/ 06604

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 5 AND 39-42  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 5 and 39-42  
are directed to a method of treatment of the human/animal  
body, the search has been carried out and based on the alleged  
effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
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restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/06604

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9222653 A	23-12-1992	AU 675916 B	27-02-1997
		AU 2250992 A	12-01-1993
		CA 2103059 A	15-12-1992
		EP 0590058 A	06-04-1994
		JP 6508267 T	22-09-1994
		WO 9404679 A	03-03-1994
<hr/>			
GB 2268744 A	19-01-1994	AT 129017 T	15-10-1995
		AT 124459 T	15-07-1995
		AT 159299 T	15-11-1997
		AU 664801 B	30-11-1995
		AU 6461294 A	22-12-1994
		AU 646009 B	03-02-1994
		AU 6974091 A	24-07-1991
		AU 649645 B	02-06-1994
		AU 7033091 A	24-07-1991
		AU 631481 B	26-11-1992
		AU 7048691 A	24-07-1991
		BG 60462 B	28-04-1995
		CA 2037607 A	07-09-1992
		CA 2046904 A	22-06-1991
		CA 2050479 A, C	22-06-1991
		DE 69020544 D	03-08-1995
		DE 69020544 T	18-01-1996
		DE 69022982 D	16-11-1995
		DE 69022982 T	28-03-1996
		DE 69031591 D	20-11-1997
		DE 69031591 T	12-03-1998
		DK 460167 T	20-11-1995
		DK 460171 T	28-08-1995
		DK 460178 T	22-12-1997
		EP 0460167 A	11-12-1991
		EP 0460171 A	11-12-1991
		EP 0460178 A	11-12-1991
		EP 0620276 A	19-10-1994
		EP 0626390 A	30-11-1994
		ES 2079638 T	16-01-1996
		ES 2074701 T	16-09-1995
		ES 2112270 T	01-04-1998
		WO 9109966 A	11-07-1991

# INTERNATIONAL SEARCH REPORT

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2268744 A		WO 9109967 A	11-07-1991
		WO 9109968 A	11-07-1991
		GB 2246781 A,B	12-02-1992
		GB 2246570 A,B	05-02-1992
		GB 2268745 A,B	19-01-1994
		GR 3017734 T	31-01-1996
		GR 3025781 T	31-03-1998
		JP 4505398 T	24-09-1992
		JP 4506458 T	12-11-1992
		JP 5500312 T	28-01-1993
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